SUMMARY OF SAFETY AND EFFECTIVENESS DATA (SSED)

I. GENERAL INFORMATION

Device Generic Name:	Real-time PCR test
Device Trade Name:	*therascreen*® KRAS RGQ PCR Kit
Device Procode:	OWD
Applicant's Name and Address:	QIAGEN Manchester Ltd. Skelton House, Lloyd Street North Manchester, UK M15 6SH
Date(s) of Panel Recommendation:	None

Premarket Approval Application (PMA) Number: P110030

Date of FDA Notice of Approval:	July 6, 2012
Expedited:	Not applicable

II. INDICATIONS FOR USE

The *therascreen* KRAS RGQ PCR Kit is a real-time qualitative PCR assay used on the Rotor-Gene Q MDx instrument for the detection of seven somatic mutations in the human KRAS oncogene, using DNA extracted from formalin-fixed paraffin-embedded (FFPE), colorectal cancer (CRC) tissue. The *therascreen* KRAS RGQ PCR Kit is intended to aid in the identification of CRC patients for treatment with Erbitux® (cetuximab) based on a KRAS no mutation detected test result.

III. CONTRAINDICATIONS

None.

IV. WARNINGS AND PRECAUTIONS

The warnings and precautions can be found in the *therascreen*® KRAS RGQ PCR Kit labeling.

V. DEVICE DESCRIPTION

The following components comprise the overall device:

- QIAGEN QIAamp® DSP DNA FFPE Tissue Kit
- QIAGEN *therascreen*® KRAS RGQ PCR Kit
- QIAGEN Rotor-Gene Q MDx, Software version 2.1.0, and KRAS Assay Package

Specimen Preparation

Formalin-fixed, paraffin-embedded (FFPE) blocks are sectioned onto glass slides. A stained slide is used to confirm that the tumor content exceeds 20% of the tissue and that a minimum tumor area of $4mm^2$ is available. A single non-stained tissue section is scraped from the slide for DNA extraction. If sections have a tumor content of less than 20%, the section should be macrodissected. DNA is manually extracted and purified from 5 µm glass-mounted sections of FFPE tissue taken from colorectal cancer patients using the QIAGEN QIAamp® DSP DNA FFPE Tissue Kit and a modified protocol. The tumor tissue is deparaffinized with xylene and the xylene is extracted with ethanol. The sample is lysed under denaturing conditions with proteinase K for one hour. The sample is heated at 90°C to reverse formalin cross-linking of genomic DNA. The sample is passed through a silica-based membrane so that genomic DNA binds to the membrane and contaminants are removed. Purified genomic DNA is eluted from the membrane into 200 µL of elution buffer. Extracted DNA is stored at -20°C.

PCR Amplification and Detection

The QIAGEN *therascreen*® KRAS RGQ PCR Kit contains reagents for eight separate reactions; seven mutation specific reactions to amplify and detect mutations in codons 12 and 13 in exon 2 of the K-Ras oncogene, and one Control Reaction that amplifies and detects a region of exon 4 in the K-Ras oncogene. Each reaction in the KRAS RGQ Kit makes use of an amplification refractory mutation system (ARMS®) allele specific polymerase chain reactions (PCR) to selectively amplify mutated genomic DNA templates (mutation-positive) in a background of non-mutated genomic DNA (mutation-negative; wild-type) combined with a fluorophore-labeled Scorpion® primer to detect any resultant amplification product. ARMS technology exploits the ability of Taq polymerase to distinguish between a match and a mismatch at the 3' end of a PCR primer. Scorpions are bifunctional molecules containing a PCR primer covalently linked to a probe. The probes incorporate both a fluorophore, [carboxyfluorescein (FAM™)] and a quencher which quenches the fluorescence of the fluorophore. During PCR, when the probe binds to the ARMS amplicon, the fluorophore and quencher become separated leading to a detectable increase in fluorescence.

Before testing with the mutation-specific test reactions, each DNA sample must be tested with the Control Reaction to determine whether the quality and quantity of DNA is sufficient and appropriate for the working range of the assay. The Control Reaction Ct value is used to assess the total amplifiable DNA in a sample and must fall within prespecified ranges for each sample.

The interpretation of the results obtained from the Control reaction is as follows:

Control Ct value	Interpretation	Action
> 32.00	Quantity of amplifiable DNA is not sufficient for mutation analysis.	Additional samples should be extracted and tested
< 21.92	Quantity of amplifiable DNA is too high for mutation analysis.	Dilute with the sample diluent water supplied in the kit
21.92 ≤ Control Ct ≥ 32.00	Quantity of amplifiable DNA is suitable for mutation analysis.	---

The run parameters used for assessing the DNA sample with the Control Reaction mix are the same run parameters for mutation analysis using the Mutation Reaction mixes. They run parameters are: (1) Hold at 95°C for 15 minutes to activate the Taq polymerase; (2) PCR for 40 cycles of 95°C for 30 seconds, to denature, and 60°C for 1 minute, to anneal/extend. The PCR cycle at which the fluorescence from a particular reaction crosses the pre-defined threshold value is defined as the Ct value. The seven mutations in codons 12 and 13 of the K-RAS oncogene detected by the Kit are listed below:

Mutation	Base Change
GLY12ALA (G12A)	GGT>GCT
GLY12ASP (G12D)	GGT>GAT
GLY12ARG (G12R)	GGT>CGT
GLY12CYS (G12C)	GGT>TGT
GLY12SER (G12S)	GGT>AGT
GLY12VAL (G12V)	GGT>GTT
GLY13ASP (G13D)	GGC>GAC

Test Controls

Each test run must contain an Internal Control, the Positive Control, and the Negative Control. A test run is considered invalid if the Negative Control indicates that the test run has been contaminated (Ct value above a set value for the FAM channel) or if the Positive Control Ct value lies outside a set range (both FAM and HEX channels).

Run Validity Criteria

Reaction Mix	Sample	RGQ Channel	Valid Ct Range*
Control	Positive Control	FAM	23.50 to 29.50
Control	No Template Control	FAM	No Amplification
Control	No Template Control	HEX	31.91 to 35.16
Mutation	Positive Control	FAM	23.50 to 29.50
Mutation	No Template Control	FAM	No Amplification
Mutation	No Template Control	HEX	31.91 to 35.16

*Ranges are inclusive

Internal Control:
All eight reactions contain an additional ARMS primer and a HEX-labeled Scorpion primer for the amplification and detection of a synthetic non K-Ras related oligonucleotide template that is used as an Internal Control. The Scorpion primer is labeled with HEX to distinguish from the FAM-labeled Scorpions in the control and mutation reactions. In each reaction, the Internal Control reaction is designed to be the weaker of the two reactions. This is achieved through the use of a very low concentration of Internal Control template. The Internal Control reaction is designed to work independently of mutation-specific amplification, but can fail in the presence of strong amplification if it is "out-competed" by the FAM reaction. A mutation negative result with a failed Internal Control reaction in any one of the seven mutation reactions will be reported as an invalid result. The Internal Control is used to detect inhibitors or gross reaction failures.

Positive Control:
The positive control is comprised of a mixture of synthetic oligonucleotides representing each of the mutations detected by the KRAS Kit. Detection of the positive control confirms the proper functioning of each of the reaction mixes in the Kit.

Negative Control:
The KRAS RGQ Kit contains nuclease-free water to be used as a no template control (NTC) reaction. The NTC serves as a negative control and assesses potential contamination during assay set up.

Instrument and Software

The Rotor-Gene Q (RGQ) MDx Instrument is a real-time PCR analyzer designed for thermocycling and real-time detection of amplified DNA. The RGQ MDx Instrument controls and monitors PCR reactions and includes the software that determines mutation status based upon PCR results. It incorporates a centrifugal rotor design for thermal cycling during PCR reactions where each tube spins in a chamber of moving air. Samples are heated and cooled in a low-mass-air oven according to a software determined cycle that initiates the different phases of the PCR cycle for a total of 40 cycles for each PCR run. In the RGQ MDx Instrument, samples are excited from the bottom of the chamber by a light emitting diode. Energy is transmitted through the thin walls at the base of the tube. Emitted fluorescence passes through the emission filters on the side of the chamber and is detected by a photomultiplier tube. Detection is performed as each tube aligns with the detection optics; tubes spin past the excitation/detection optics every 150 milliseconds. The fluorescence signals monitor the progress of the PCR reactions. The instrument is capable of supporting up to six optical channels (six excitation sources and six detection filters), however only two of these channels (the FAM and HEX channels) are used with the KRAS Kit.

The *therascreen* KRAS Assay Package consists of two templates: the "therascreen KRAS QC Locked Template" (for DNA sample assessment) and the "therascreen KRAS Locked Template" (for detection of KRAS mutations).These templates contain the PCR run

parameters and calculate the results. The same run parameters are used for both the DNA sample assessment with the Control Reaction Mix and for detection of KRAS mutations using the mutation reaction mixes.

The RGQ MDx Instrument software supports real-time analysis procedures. The software determines Ct values, calculates ΔCt values, and compares these to the mutation-specific cut-off values incorporated into the software as described above. A system of Flags/Warnings is embedded within the software in order to inform the user of potential problems with the assay and to indicate non-valid test runs or non-valid samples within a valid test run (inappropriate level of DNA or Internal Control failure). No results are reported for invalid runs or for non-valid samples. Users of the KRAS RGQ Kit cannot make subjective determinations of mutation status as they do not have access to the Ct or ΔCt values and only see the mutation status calls reported by the software.

Interpretation of Results

The Ct for the control reaction reflects the total amount of amplifiable K-Ras template in the sample, while the Ct for the allele specific reactions reflect the amount of K-Ras mutation within the sample. The difference in Ct values (ΔCt) between the control reaction and the allele-specific reaction indicates the proportion of mutation within the sample. The ΔCt value approaches 0 as the proportion of mutant DNA in the samples increases. The ΔCt value increases (approaches the threshold for positive vs. negative call) as the proportion of mutant DNA in the sample decreases. When the ΔCt measure exceeds ΔCt cut-off values for the mutant reactions, the assay reports no mutation detected (e.g., negative for the 7 mutations).

For each sample, a calculation is performed by the RGQ MDx Instrument software to determine the ΔCt value (FAM channel) for each of the 7 mutation-specific reactions:

[Mutation reaction Ct value] – [Control Reaction Ct value] = ΔCt

Based on pre-determined analytical Ct and ΔCt values, the Rotor-Gene Q software qualitatively determines the mutation status of the DNA samples and reports which samples contain which mutation. Each sample will have seven possible ΔCt values (one per mutation). These values are compared to pre-established specifications (cut-off values) incorporated into the RGQ MDx Instrument software to determine whether a sample is mutation positive or negative and which mutation, if any, is present. When the mutation reaction ΔCt value is less than or equal to the cut-off value for that reaction, the sample is K-Ras mutation-positive. The assay results will be displayed as "Mutation Positive," "No Mutation Detected," "Invalid" or, if a run control fails, "Run Control Failed." For the mutation-positive samples, specific mutations are reported.

Mutation Assay	**12ALA**	**12ASP**	**12ARG**	**12CYS**	**12SER**	**12VAL**	**13ASP**
Cut-Off (ΔCt)	**≤ 8.0**	**≤6.6**	**≤8.0**	**≤8.0**	**≤8.0**	**≤7.5**	**≤7.5**

VI. ALTERNATIVE PRACTICES AND PROCEDURES

There are no other FDA-cleared or approved alternatives for the testing of colorectal cancer tissue for detecting mutations in the K-Ras oncogene for the selection of patients who may benefit with Erbitux® (cetuximab) therapy.

VII. MARKETING HISTORY

The QIAGEN *therascreen*® KRAS RGQ PCR Kit has not been marketed in the United States or any foreign country.

VIII. POTENTIAL ADVERSE EFFECTS OF THE DEVICE ON HEALTH

Failure of the device to perform as expected or failure to correctly interpret test results may lead to incorrect K-Ras test results, and consequently improper patient management decisions in colorectal cancer treatment. A false positive test result may lead to Erbitux® (cetuximab) treatment being withheld from a patient who might have benefitted. A false negative test result may lead to Erbitux® (cetuximab) treatment being administered to a patient who is not expected to benefit, and potentially any adverse side effects associated with treatment. For the specific adverse side effects that are associated with Erbitux® (cetuximab) treatment, please see Section X below.

IX. SUMMARY OF PRECLINICAL STUDIES

A. Laboratory Studies

The specific performance characteristics of the QIAGEN *therascreen*® KRAS RGQ PCR Kit (henceforth referred to as KRAS Kit) were determined by studies using formalin-fixed, paraffin-embedded (FFPE) tissue specimens collected from colorectal cancer patients (CRC) and 8 formalin-fixed, paraffin-embedded human cell lines (FFPE cell lines) of which 7 harbor known K-Ras mutations, and one K-Ras wild-type (i.e., glycine amino acids at codons 12 and 13). Mutation status of specimens was confirmed by bi-directional Sanger sequencing. The similarity between FFPE cell lines and FFPE clinical specimens was demonstrated. FFPE cell lines were sectioned and processed similar to FFPE patient specimens. DNA was extracted and tested according the instructions for use.

1. Comparison to Reference Method

To demonstrate the accuracy of the KRAS Kit relative to Sanger bi-directional sequencing, two accuracy studies with procured specimens were conducted. In the first study, a set of 350 procured tumor specimens from CRC patients was obtained based on characteristics that aligned with patient samples screened in the clinical trial (i.e., intended use population). Variables that impact test performance were described for the procured specimens and compared to the clinical trial specimens. The variables were deemed similar between both groups and consisted of patient demographics (age, genders, race, and country of origin), fixation process, tumor sampling, tumor tissue content, stage, histology, amount of necrotic tissue, and storage conditions. Using a statistical random sampling technique, 150 samples of unknown mutation status were chosen for evaluation. Ten sequential 5µm sections

were cut from each sample and mounted onto glass slides. Sections used for testing with the KRAS Kit or for bi-directional sequencing were adjacent to each other. Specimens were processed and tested by the KRAS Kit according to the final product labeling. All sections used for bi-directional sequencing were macrodissected to enrich for tumor content. A Phred score greater than 40 was a pre-specified acceptance criterion for sequencing (seven samples that failed were excluded). Invalid and indeterminate samples were retested according to protocol. The results demonstrated that the KRAS Kit reported two samples as negative. These samples were reported by bi-directional sequencing to be positive for 12ASP or 13ASP. In contrast three samples were reported as having a K-Ras mutation by the KRAS Kit that were not reported as positive by sequencing. In addition, one sample identified as 12ARG by the KRAS Kit was determined to be 12ASP by sequencing. The overall results are shown in the Table below.

KRAS Kit compared to Sanger bi-directional Sequencing

		Mutation Call by Bi-Directional Sequencing									
		Mutation Negative	**IND**	**12ALA**	**12ARG**	**12ASP**	**12CYS**	**12SER**	**12VAL**	**13ASP**	**Total**
KRAS Kit call	**Indeterminate**	3	-	-	-	-	-	-	-	-	3
	Invalid	2	-	-	-	-	-	-	-	-	2
	Mutation-negative	80	-	-	-	1	-	-	-	1	82
	12ALA	-	-	3	-	-	-	-	-	-	3
	12ARG	-	-	-	-	1	-	-	-	-	1
	12ASP	-	-	-	-	20	-	-	-	-	20
	12CYS	-	-	-	-	-	3	-	-	-	3
	12SER	-	1	-	-	-	-	-	-	-	1
	12VAL	2	-	-	-	-	-	-	14	-	16
	13ASP	1	-	-	-	-	-	-	-	11	12
	Total	88	1	3	0	22	3	0	14	12	143

Concordance between methods for this study was calculated as the positive percent agreement (PPA), negative percent agreement (NPA) and overall percent agreement (OPA) with the 95% confidence intervals for all samples with valid results. The results demonstrate a PPA of 96.3%, a NPA of 96.3% and an OPA of 96.4%.

Agreement for Samples with both Sanger and KRAS Kit valid Results

Measurement of Agreement	Percentage	95% CI
Overall percent agreement (OPA)	96.3% (132/137)	92.69 – 98.21
Percent positive agreement (PPA)	96.3% (52/54)	89.41 – 98.77
Percent negative agreement (NPA)	96.4% (80/83)	91.30 – 98.55

A sensitivity analysis was performed to evaluate the agreement between the two methods if all of the KRAS Kit indeterminate and invalid results were treated as mutation-positive or as mutation-negative. Under the condition where all of the invalid/indeterminate calls are assumed to be positive, the NPA was reduced to 90.9% and the OPA reduced to 92.9%.

A second unique set of 271 CRC FFPE specimens were procured and compared to Sanger bi-directional sequencing as described above to supplement the data from the first study. The set consisted of 250 specimens of unknown mutations status, and 21 specimens of known mutation status to enrich for rare mutations. A total of 13 (~5%) specimens required macrodissection in accordance with KRAS Kit instructions because the tumor content was less than 20%. Out of the 271 specimens tested, 24 were indeterminate (failed control Ct range). Concordance analysis was carried out on 247 samples with both valid bi-directional and KRAS Kit results. There were 9 discordant samples. One sample from the 247 samples had a mutation positive result with bi-directional sequencing but a mutation negative result with the KRAS Kit. Eight samples were shown to have a positive result with the KRAS Kit but a negative result with bi-directional sequencing. The results are shown in the tables below. Overall agreement was 96.4%. The data supports the performance of the *therascreen*® RGQ PCR KRAS Kit.

Comparison of Mutation Calls by KRAS Kit and Bi-directional Sequencing

		Mutation Call by Bi-Directional Sequencing									
		Mutation Negative	**IND**	**12ALA**	**12ARG**	**12ASP**	**12CYS**	**12SER**	**12VAL**	**13ASP**	**Total**
KRAS Kit call	**Indeterminate**	15	5	1	-	1	1	-	1	-	24
	Mutation-negative	132	-	-	-	-	-	1	-	-	133
	12ALA	-	-	10	-	-	-	-	-	-	10
	12ARG	5	-	-	5	-	-	-	-	-	10
	12ASP	-	-	-	-	31	-	-	-	-	31
	12CYS	1	-	-	-	-	11	-	-	-	13
	12SER	-	-	-	-	-	-	13	-	-	13
	12VAL	2	-	-	-	-	-	-	25	-	27
	13ASP	-	-	-	-	-	-	-	-	11	11
	Total	155	5	11	5	32	12	14	26	11	271

Agreement between Sanger and KRAS Kit*

	Percent observed agreement (Lower 95% confidence interval)
Overall percent agreement	96.36% (93.73%)
Positive percent agreement	99.07% (95.64%)
Negative percent agreement	94.29% (89.93%)

* Excluding the 24 KRAS Kit indeterminate results (failed Control Ct range)

2. Analytical Sensitivity

a) Limit of Blank (LoB) – No Template

To assess performance of the *therascreen*® KRAS RGQ PCR Kit in the absence of template and to ensure that a blank sample does not generate an analytical signal that might indicate a low concentration of mutation, samples with no template were evaluated. Ten KRAS Kit runs consisting of specimens with nuclease-free water (no DNA template) were conducted. Each of the runs included positive and negative controls, as well as seven no-template samples. The results demonstrated no detectable control or mutant Ct values in any of the mutation or control reaction wells (Internal control Ct values were all valid). Results are reported as invalid due to failed controls in the absence of DNA.

b) Limit of Detection (LoD)

The *therascreen*® KRAS RGQ PCR Kit does not use a specific concentration of DNA as determined by spectrophotometry. DNA input is based on the Control reaction Ct result which is used to indicate that there is sufficient amplifiable DNA present in the sample. The stated DNA input for the assay is defined by the Control Ct prespecified range 21.92 to 32.00. For the *therascreen*® KRAS RGQ PCR Kit, the limit of detecting mutant DNA in a background of wild-type DNA is defined as the lowest dilution factor at which 95% of the test replicates for each mutation positive sample were determined to be positive. Eight FFPE cell lines; seven with known mutant DNA content and one wild-type were used for this evaluation. The proportion of mutant in total amplifiable DNA (percent mutant DNA) was determined previously using a bi-directional Sanger sequencing method from unfixed cells followed by relative peak analysis. In the case of three cell lines the mutant content was 100% (i.e., the cell line DNA was homozygous mutant). The other cell lines were of mixed zygosity. Multiple DNA extractions from each sample were pooled to generate DNA stocks. The DNA stocks were then normalized to achieve target Control reaction Ct values. Normalized mutant DNA extracts were diluted with normalized WT DNA extract to create a dilution series of extracts containing the same level of total amplifiable DNA but different levels of mutant DNA. Serial dilutions were then generated from these samples and 9 replicates for each dilution were run. The first dilution series was created for the mid-range Control reaction Ct value (approximately 26). The percentage of correct calls as a function of the dilution for each mutant reaction is shown below. Shaded boxes indicate the corresponding dilution for each mutant reaction in which greater than 95% of the replicates produced correct calls.

Percentage of Correct Calls

	% Correct Calls						
% Mutation Dilution	**12ALA**	**12ASP**	**12ARG**	**12CYS**	**12SER**	**12VAL**	**13ASP**
0.78	100	0	33.3	55.6	22.2	66.7	0
1.56	100	33.3	100	100	88.9	100	0
3.13	100	77.8	100	100	100	100	66.7
6.25	100	100	100	100	100	100	100
12.5	100	100	100	100	100	100	100
25.0	100	100	100	100	100	100	100
50.0	100*	100	100	100	100	100	100

* Mutation Dilution for this sample was 40.0.

The results of the first dilution series were used to generate dilutions for the confirmation of LoD values using narrower, reaction-specific ranges of percent mutation dilutions at both low and high levels within the input range of the assay. The target value for the High series was approximately Ct 23-24. Twelve replicates for each dilution were evaluated for the High dilution series. The percentage of correct calls is described for each dilution in the High series and is shown in the tables below. Shaded boxes indicate the percentage in which greater than 95% of the replicates produced correct calls.

Percentage of Correct Calls for High Dilution Series

		% Mutation Dilution (High)					
12ALA		0.13	0.27	0.54	**1.08**	2.15	4.30
	% correct calls	0	0	91.7	**100**	100	100
12ASP		0.56	1.13	2.25	4.50	**9.00**	18.00*
	% correct calls	0	8.3	33.3	83.3	**100**	100
12ARG		0.16	0.33	0.65	**1.30**	2.60	5.20
	% correct calls	0	0	8.3	**100**	100	100
12CYS		0.12	0.24	0.49	0.98	**1.95**	3.90
	% correct calls	0	0	8.3	83.3	**100**	100
12SER		0.31	0.63	1.25	2.50	**5.00**	10.00
	% correct calls	0	0	33.3	66.7	**100**	100
12VAL		0.17	0.34	0.69	**1.38**	2.75	5.50
	% correct calls	0	0	16.7	**100**	100	100
13ASP		0.63	1.25	2.50	**5.0**	10.0	20.0
	% correct calls	0	0	0	**100**	100	100

*Eleven valid replicates in this evaluation

The target Ct value for the Low series was approximately 31. Each dilution was run as 24 replicates unless otherwise indicated. The percentage of correct calls is described for each dilution in the Low series and is shown in

the tables below. Shaded boxes indicate the percentage in which greater than 95% of the replicates produced correct calls.

Percentage of Correct Calls for Low Dilution Series

		% Mutation Dilution (Low)						
12ALA		0.27	0.54	1.08	2.15	**4.30**	8.60	12.90
	% correct calls	12.5	20.8	33.3	83.3	**100**	100	100
12ASP		0.56	1.13	2.25	4.50	**9.0**	18.0	27.0
	% correct calls	0	16.7	29.2	58.3	**100**	100	100
12ARG*		0.33	0.65	1.30	2.60	**5.20**	10.4	15.6
	% correct calls	8.3	4.2	29.2	52.2	**95.8**	100	100
12CYS		0.24	0.49	0.98	1.95	3.90	**7.80**	11.7
	% correct calls	8.3	4.2	20.9	54.2	83.3	**100**	100
12SER		0.63	1.25	2.50	5.0	10.0	20.0	**30.0**
	% correct calls	0	0	8.3	33.3	70.9	83.3	**100**
12VAL**		0.34	0.69	1.38	2.75	**5.50**	11.00	16.50
	% correct calls	4.3	16.7	46.7	75.0	**100**	100	100
13ASP		0.63	1.25	2.5	5.0	10.0	**20.0**	30.0
	% correct calls	0	4.2	8.3	33.3	70.8	**100**	100

*For the 2.60 dilution, the number of valid replicates was 23 for 12ARG.
**Valid replicates for the 12VAL series were 23, 24, 15, 16, 13, 12, and 19.

Logistic Regression models were applied to each assay individually for the low and high input DNA datasets. In these models, the response variable was the binary output of mutation detected (detect = 1) and mutation not detected (detect = 0), the continuous explanatory variable was $\log_2$ % mutation dilution. The LoDs were calculated as the percent mutation dilution which gave a predicted probability of detection of 0.95. The LoDs determined from the dilution series beginning with either the low or high Ct values are shown in the Table below.

Logistics Regression Data for Low and High Ct Dilution Series

	Low	**High**
12ALA	4.25	0.56
12ASP	7.27	0.87
12ARG	10.23	6.43
12CYS	6.90	1.21
12SER	25.75	4.20
12VAL	5.17	0.90
13ASP	18.83	4.16

The data overall supports the manufacturers Final LoD claims for FFPE cell lines when the input Ct value is between approximately 22 and 27 Ct. At the lower end of the Ct input range, the sensitivity of the assay decreases as the amount of input DNA may not contain sufficient copies to support the same percentage ratios of wild type to mutant DNA observed within the high and mid points of the working range.

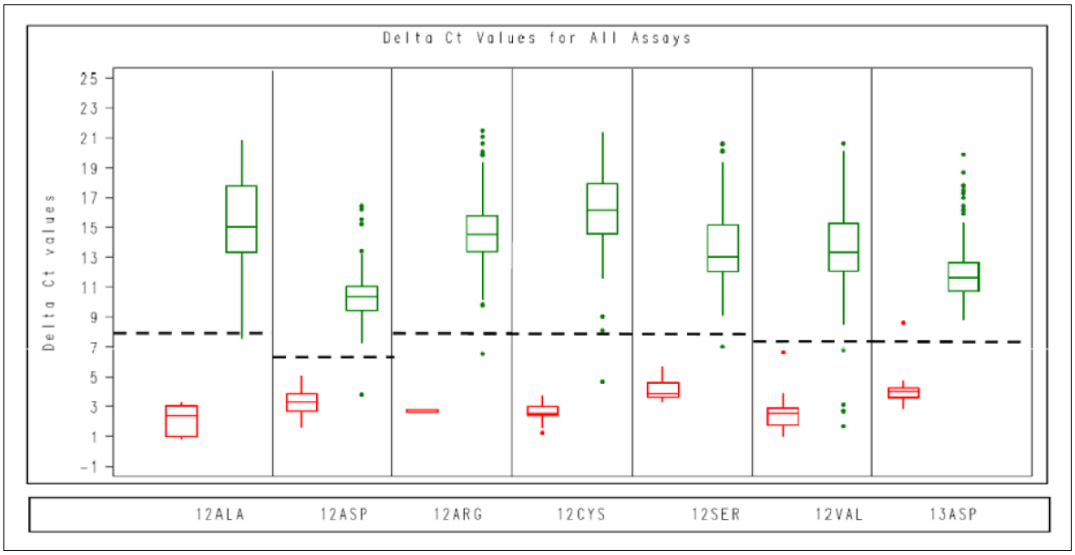
LoD Claims FFPE Cell Lines when Control Reaction Ct Range ~22-27

Mutation Reaction	Final LoD Claim
12ALA	**0.8**
12ASP	**2.6**
12ARG	**6.4**
12CYS	**1.5**
12SER	**5.6**
12VAL	**1.6**
13ASP	**6.4**

c) <u>Control Ct Range and RFI Validation</u>

For the KRAS Kit, the acceptable Control Ct range that is used to determine the amount of DNA input and the threshold value is set at 0.05 relative fluorescence units (RFI). This value is configured in the KRAS Locked Templates for both the FAM and HEX channels. The threshold value and Control Ct range were defined during development of the KRAS Kit. Briefly, 220 FFPE samples were assayed. Control reaction Ct values were tested for normality and one-side tolerance limits were developed such that 97.5% of the Control Ct values in the mutation reactions fall above the lower limit with a confidence level >90%. Box and Whisker plots of the ΔCt values were based on the assignment of the optimized Control Ct range and are shown in the box below. The line within each box represents the median value in the distribution, while the box represents the 25th and 75th percentile. The hashed line shows the cut-offs, above that are the mutation negative samples, and below that the mutation-positive samples.

Cut-off ΔCt Values by Mutation



3. Effect of DNA Input on ΔCt Values

The DNA input level is defined as the total quantity of amplifiable K-Ras DNA in a sample and is determined by the Ct values from the Control reaction. When samples at different total DNA levels contain the same proportion of mutant DNA, it is expected that the measured ΔCt values will remain consistent. The objective of the study was to demonstrate that the performance of the KRAS Kit is consistent over the total DNA input (Control Ct) range of the assay. DNA extracted from 8 FFPE cell lines was used to prepare pools of DNA with the lowest achievable control reaction Ct. Concentrated DNA stocks were subsequently diluted to generate DNA spanning the working range (total of 5 dilutions including the initial concentrated stock). For mutations 12ALA, 12ASP, 12ARG and 12VAL were diluted 1:10 (range 1 to .0001). For mutations 12CYS, 12SER and 13ASP, the dilutions were 1:5.5 (range 1 to 0.001). For each point within the working range sufficient material was prepared to carry out 6 replicate tests. The Dilution range for each mutation reaction, mean Ct value for the Control and Ct reactions in the test runs are shown in the Table below. The results from each mutation pool tested showed Control Reaction Ct values corresponding with the targeted values. For each of the mutations detected by the KRAS Kit, the ΔCt values measured at different total DNA input levels spanning the working range of the assay passed the pre-set acceptance criteria for the study.

Mean Ct Values across the Control Reaction Ct Range – FFPE Cell Lines

	Mean Mutation Ct Value					Mean Control Ct Value				
	Dilution 1	2	3	4	5	Dilution 1	2	3	4	5
12ALA	22.75	25.57	28.77	32.31	35.77	21.19	24.32	27.61	31.17	34.51
12ASP	23.45	26.32	29.64	33.28	36.5	20.99	24.14	27.54	31.17	34.66
12ARG	21.37	24.71	28.05	31.49	34.84	20.19	24.08	26.97	30.55	33.78
12CYS	24.32	26.64	28.99	31.45	33.8	23.35	25.94	28.4	30.64	33.13
12SER	25.54	27.19	29.67	32.14	34.61	22.63	24.98	27.52	29.99	32.53
12VAL	21.53	24.72	28.05	31.24	34.41	21.24	24.47	27.9	30.99	34.51
13ASP	26.7	28.54	30.8	33.12	35.65	23.13	25.7	28.26	30.66	33.03

The mean ΔCt values for each mutation reaction and each dilution are displayed as a function of the Control Reaction Ct ranges for the dilutions in the Table below. Although there is a slight increase in ΔCt as DNA input increases, overall, the ΔCt values were consistent across the working range of the KRAS Kit within the prespecified acceptance criteria.

Mean ΔCt Values across the Control Reaction Ct Range - FFPE Cell Lines

	Dilution (1) ~20-21Ct	Dilution (2) ~ 23-24Ct	Dilution (3) ~ 26-27Ct	Dilution (4) ~29-30Ct	Dilution (5) ~32-33Ct
	Mean ΔCt				
12ALA	1.56	1.25	1.16	1.14	1.27
12ASP*	2.46	2.18	2.11	2.11	1.75
12ARG	1.18	0.63	1.08	0.94	1.06
12VAL	0.29	0.25	0.15	0.26	-0.1
	~ 22-23Ct	~ 24-25Ct	~ 27-28Ct	~29-30Ct	~32-33Ct
	Mean ΔCt				
12SER	2.91	2.21	2.15	2.15	2.08
12CYS	0.98	0.71	0.58	0.81	0.67
13ASP	3.57	2.84	2.54	2.46	2.62

*In the case of 12ASP data, total number of replicates was 27.

DNA extracts from FFPE CRC samples were also prepared and diluted to represent three total DNA input levels; nominally High, Medium and Low DNA input levels as defined by the absolute Ct value of the KRAS Kit Control reaction. The High and Medium DNA input levels were within the working range of the assay (i.e., Control Ct range 21.92 – 32.00). The Low DNA input level dilutions were targeted to be within the working range of the assay, however, for the purposes of this study, values that fell outside the working range were also included in the study analysis. The data demonstrates ΔCt values are similarly consistent across DNA input levels.

Effect of DNA Input on ΔCt Values across the Input Ct Range - CRC Samples

	Mean Control Ct			Mean Mutant Ct			Mean ΔCt		
	Low	**Med**	**High**	**Low**	**Med**	**High**	**Low**	**Med**	**High**
12ALA	31.50	28.36	25.41	33.19	30.14	27.37	**1.69**	**1.78**	**1.95**
12ASP	30.74	27.52	25.44	33.00	29.90	27.16	**2.26**	**2.39**	**2.72**
12ARG	32.12	29.23	27.03	34.18	31.34	29.24	**2.06**	**2.11**	**2.21**
12CYS	30.85	27.36	24.43	33.51	30.10	27.43	**2.66**	**2.74**	**3.00**
12SER	30.35	27.64	25.17	35.04	32.11	30.13	**4.70**	**4.47**	**4.96**
12VAL	30.97	27.48	24.39	32.97	29.60	26.54	**2.00**	**2.12**	**2.16**
13ASP	31.14	27.77	25.13	34.58	31.26	29.05	**3.44**	**3.49**	**3.92**

4. Linearity/Amplification Efficiency as a Function of DNA Input (Part 1)

The linearity and amplification efficiency of PCR for each mutation reaction, relative to the control reaction, across the working range of the KRAS Kit was demonstrated. Amplification efficiency was calculated for each of the mutation reactions and the control reaction as [2(-1/slope)]-1. The largest difference in the amplification efficiencies between the Control reaction and a mutant reaction was observed for the 13ASP (mean difference in efficiencies approximately 14.5%). The amplification efficiency of the control relative to the mutant reactions is consistent across the working range of the assay as shown in the Table below.

PCR Amplification Efficiencies (Part 1)

			Intercept	Intercept Standard Error	Calculated Slope	Standard Error (slope)	Two-Sided 95% CI (slope)	**Amplification Efficiency**	Difference in Amplification Efficiencies
Sample	**12ALA**	**Control Ct**	21.06	0.060	-1.008	0.007	-1.023, -0.993	0.989	0.03
		12ALA Ct	22.48	0.103	-0.987	0.013	-1.013, -0.961	1.019	
	12ASP	**Control Ct**	20.82	0.083	-1.035	0.01	-1.056, -1.014	0.954	0.056
		12ASP Ct	23.24	0.083	-0.993	0.011	-1.016, -0.97	1.01	
	12ARG	**Control Ct**	20.38	0.13	-1.013	0.016	-1.046, -0.98	0.982	-0.003
		12ARG Ct	21.35	0.065	-1.015	0.008	-1.032, -0.999	0.979	
	12CYS	**Control Ct**	23.43	0.063	-0.981	0.01	-1.003, -0.96	1.026	0.032
		12CYS Ct	24.29	0.039	-0.961	0.006	-0.974, -0.947	1.058	
	12SER	**Control Ct**	22.57	0.050	-1.003	0.008	-1.02, -0.986	0.996	0.105
		12SER Ct	25.21	0.087	-0.934	0.014	-0.963, - 0.904	1.101	
	12VAL	**Control Ct**	21.21	0.047	-0.995	0.006	-1.007, - 0.983	1.007	0.033
		12VAL Ct	21.53	0.043	-0.972	0.005	-0.983, - 0.961	1.04	
	13ASP	**Control Ct**	23.21	0.056	-1.001	0.009	-1.02, -0.982	0.999	0.145
		13ASP Ct	26.47	0.106	-0.909	0.017	-0.945, -0.873	1.144	

5. Linearity/Amplification Efficiency as a Function of %Mutation (Part 2)

The objective of this study was to evaluate the linearity of each mutant reaction across the working range of the assay, when the total amount of DNA is held constant but the percentage of mutant DNA is varied. DNA extracts from FFPE cell lines were initially assessed by OD readings prior to carrying out PCR with the KRAS Kit. DNA stocks were then prepared to a Control Reaction Ct corresponding to ~23Cts. The stocks were diluted serially 2 fold each time using wild-type DNA, in order to maintain the total wild-type DNA constant while varying the percentage mutant DNA in the template. Thus, each of the templates generated had the same absolute quantity and concentration of DNA but differing ratios of wild-type to mutant DNA. The dilutions and Target Ct values are shown below.

Mean Control Reaction Ct Values for Each Dilution Point

Dilution (x100)	Assay	12ALA	12ASP	12ARG	12CYS	12SER	12VAL	13ASP
		Target 22.75	Target 22.75	Target 22.75	23.17	Target 23.00	Target 22.75	Target 23.5
1	**control**	22.33	22.87	22.97	23.68	22.76	22.29	23.51
0.5	**control**	22.42	22.71	22.98	23.45	22.98	22.60	23.75
0.25	**control**	22.51	22.62	22.95	23.40	23.05	22.61	23.75
0.125	**control**	22.54	22.62	22.90	23.36	23.11	22.65	23.65
0.0625	**control**	22.55	22.66	22.90	23.29	23.08	22.67	23.64

Pools of DNA sufficient for 6 replicates per mutation were prepared. The Ct and ΔCt data for each mutation at each dilution point were calculated. The control reaction Cts were consistent over the dilution series of each mutation. For each sample where the control reaction Ct value fell within the specified range (21.92 – 32.00), ΔCt values were calculated. A linear regression model was fitted with mutation reaction Ct versus $\log_2$ DNA input dilution. The slope and 95% confidence intervals were reported. The study showed the dilution of mutations in a background of a constant concentration of wild type DNA resulted in amplification efficiencies that did not vary significantly outside the values determined in the above linearity study with the amplification efficiencies.

PCR Amplification Efficiencies (Part 2)

	Intercept	Intercept (Std. error)	Slope	95% CI (slope)	Amplification Efficiency
12ALA	23.5	0.025	-0.968	-0.989, -0.947	1.047
12ASP	24.8	0.054	-1.030	-1.075, -0.985	0.960
12ARG	24.2	0.028	-1.008	-1.031, -0.984	0.990
12CYS	24.4	0.027	-0.981	-1.003, -0.959	1.024
12SER	25.4	0.054	-0.892	-0.937, -0.847	1.174
12VAL	22.7	0.035	-1.021	-1.050, -0.992	0.972
13ASP	27.6	0.057	-0.810	-0.857, -0.763	1.353

6. Analytical Specificity

a) Primer and Probe Specificity

The primers and probes have been designed to avoid any known K-Ras polymorphisms. A specificity analysis was conducted using the Basic Local Alignment Search Tool (BLAST) to ensure that the primers used in the *therascreen*® KRAS RGQ PCR Kit would amplify only human K-Ras sequences and not sequences from other species or to non-K-Ras human sequences (e.g., pseudogenes). No non-specific amplification is predicted from non-K-Ras genes. In addition, alignments of pairs of oligonucleotides (primers, probes, and templates) used in the KRAS Kit were performed to ensure there is no unexpected binding that could lead to non-specific amplification. There was no significant homology between the various reagents.

b) Cross-Reactivity/Exclusivity

The *therascreen*® KRAS RGQ PCR Kit is comprised of eight separate reactions; one single control reaction that detects a nonpolymorphic region of the K-Ras gene and seven mutation specific reactions. There is no reaction that specifically measures the wild-type K-Ras sequence at codon 12 or 13. The K-Ras mutation-negative result, (generally wild-type at codon 12 and 13) is determined from the absence of any of the 7 mutations resulting in a positive mutation result. Therefore it is necessary to demonstrate the amount of non-specific amplification, or cross-reactivity that occurs in each reaction with excess amounts of K-Ras wild-type DNA, to ensure no false positive results occur. Similarly, non-specific amplification of KRAS mutations for which the reaction is not intended to detect is evaluated to demonstrate that the amount of cross-reactivity between mutant reactions does not result in erroneous mutation calls in the presence of excess amounts of mutant DNA. Since the DNA input for this assay is based on the control Ct range (21.92 to 32.00), the highest concentration of DNA input is based on having a control Ct value of approximately 22. FFPE clinical samples were used for this evaluation, however due to the difficulty of obtaining DNA at the maximum input level, FFPE cell line DNA was also evaluated. Mutation status was confirmed by bi-directional sequencing.

Non-Specific Amplification/Cross-Reactivity: Wild-Type K-Ras DNA:

In order to address the amount of non-specific amplification of wild-type (WT) DNA by reaction mixes designed to amplify specific mutations, sixty (60) replicates of WT FFPE cell line DNA, or DNA extracted from CRC tumor tissue at the highest concentration of amplifiable DNA input level, was evaluated using the KRAS Kit. For DNA extracted from FFPE cell line the Control Ct values were approximately 22-23. Control Ct values for three wild-type CRC samples were between 24 and 25. The results demonstrated that the ΔCt Values exceeded the established cut-offs. The mean and/or lowest ΔCt values observed for each reaction is shown below.

Lowest Mean ΔCt Observed for Wild type samples in Mutant Reactions

		WT FFPE cell line	WT Clinical CRC Samples		
Mutant Reaction	**Cut-off**	**Lowest ΔCt Observed**	**Sample 1 ΔCt Mean (Lowest)**	**Sample 2 Mean (Lowest)**	**Sample 3 Mean (Lowest)**
12ALA	**8**	12.76	18.00 (11.40)	18.62 (11.50)	20.03 (19.36)
12ASP	**6.6**	10.35	10.90 (9.62)	10.34 (8.84)	10.68 (9.01)
12ARG	**8**	14.26	20.33 (12.94)	20.02 (13.20)	20.03 (19.36)
12CYS	**8**	13.66	20.62 (17.38)	20.29 (19.62)	20.03 (19.36)
12SER	**8**	11.97	17.26 (11.14)	17.90 (11.42)	18.05 (10.44)
12VAL	**7.5**	11.81	14.87 (11.46)	16.27 (11.50)	18.68 (11.36)
13ASP	**7.5**	10.94	12.35 (9.08)	13.68 (10.69)	14.82 (9.97)

Non-Specific Amplification/Cross-Reactivity/Exclusivity: Mutation-Positive K-Ras DNA:

The exclusivity of the KRAS Kit is intended to discriminate between mutation negative and mutation positive status. Mutant samples that have a high concentration of input DNA were tested against all reaction mixes by preparing DNA samples from each of the FFPE cell lines so that the Control Reaction Ct corresponded to approximately 23. Six (6) replicates of each mutation sample were evaluated. The percentage of mutation in the sample was governed by the percentage of mutant in the cell line DNA. The mean ΔCt are presented in the table below and demonstrates that there is cross reactivity between mutant reactions when high concentrations of DNA are evaluated. The 12ALA mutation was amplified and generated ΔCt values below the ΔCt thresholds for the 12CYS, 12SER and 12VAL reactions. The 12VAL mutation was amplified and generated a ΔCt value below the ΔCt threshold for the 12ALA reaction, however in all cases, the results demonstrate that the correct mutation was called with the matched mutation reaction (i.e., the smallest ΔCt value was the correct mutation call). All other test cases were either not detected or outside the ΔCt threshold.

Cross-Reactivity Between Mutation Reactions Using FFPE Cell Line DNA(1)

		ΔCt Cut-Off	Mutant Reaction ΔCt Values						
			12ALA	**12ASP**	**12ARG**	**12CYS**	**12SER**	**12VAL**	**13ASP**
Mutant DNA	**12ALA**	8	**1.42**	12.66	-	5.81	2.78	6.31	13.21
	12ASP	6.6	12.56	**2.42**	-	-	13.44	11.21	13.55
	12ARG	8	13.12	11.56	**1.12**	11.42	-	13.43	12.66
	12CYS	8	14.2	12.48	9.23	**0.98**	-	7.96	12.88
	12SER	8	-	13.39	13.31	-	**3.02**	12.99	13.97
	12VAL	7.5	6.83	-	-	-	13.38	**0.28**	13.74
	13ASP	7.5	-	13.29	13.89	-	-	14.36	**4.5**

(1) ΔCt from matched reactions are shown in bold. Blank cells show no cross-reaction. ΔCt from cross-reactive reactions below the cut-off are shaded.

7. Interference Effects - Necrotic Tissue

To support the performance of the KRAS Kit with tissues with high necrosis, the accuracy of the KRAS Kit for 29 samples with greater than 50% necrosis as determined by a pathologist was evaluated. The KRAS Kit did not falsely detect any mutations in the 21 samples identified as wild-type by bi-directional sequencing. The KRAS Kit correctly called 7 of the 8 K-Ras mutant samples. One sample was invalid due to inadequate DNA in the sample. The results support the use of the KRAS Kit with samples with high necrosis.

8. Interference Effects - Exogenous Substances

To evaluate the impact of interfering substance on performance of the KRAS Kit, potentially interfering substances present in the DNA extraction process, were tested at 10x concentration in mutant and wild-type samples with a target Ct value between 27 and 30. The impact of each substance on the ΔCt values and mutation status of the samples was assessed. The substances tested were (1) paraffin wax, (2) xylene, (3) ethanol, and (4) Proteinase K. The difference between the ΔCt of samples with interferent was compared to samples without interferent according to statistical methods outlined in CLSI guidance document EP7-A2. For mutant samples, of the 448 replicates tested (7 mutations x 8 substances x 2 levels X 4 replicates) there were 8 invalid or indeterminate results and 4 false mutation negative results. For wild type samples, 3 of the 448 replicates tested were invalid or indeterminate and 2 false mutation positive results. The results demonstrated that there was no significant interference.

9. Reproducibility

The repeatability and reproducibility of the KRAS Kit was investigated by testing DNA extracted from 8 FFPE CRC tissue blocks representing the 7 K-Ras mutations and one wild-type (WT) K-Ras specimen, at three sites with 2 operators at each site across 5 non-consecutive days in duplicate. The study consisted to two-parts: within-laboratory and between-laboratory. Multiple 5-µm sections were cut from each of the blocks and mounted onto glass slides. DNA was extracted using the QIAGEN® QIAamp® DSP DNA FFPE Tissue Kit, pooled and diluted to produce stocks of the same concentration of amplifiable DNA based on target control Ct values. Aliquots of mutant DNA stocks were diluted with WT stock to produce individual DNA samples at the targeted Ct levels representing approximately 3 times the LoD and at the cut-off. (The cut-off sample tested was one that was designed to investigate the variance directly at the cut-off.) The total set consisted of 21 samples; 7 mutation-positive samples at 3xLoD, 7 mutation-positive samples at the cut-off (i.e., at ΔCt cut-off to investigate variance at the cut-off), and 7 wild-type samples.

The within-laboratory precision was determined from a series of 20 runs evaluating both dilution levels and a duplicate on each run resulting in 40 replicates per sample (2 operators and 5 non-consecutive days). Between-laboratory reproducibility was evaluated at two additional sites using 2 operators and 5 non-consecutive days in duplicate resulting in 10 runs and 20 duplicates per sample. Three lots were used in this evaluation. The proportion of correct

mutation call of 3xLoD samples testing mutant and WT samples were reported.

The estimated proportion of 3xLOD samples testing mutant and WT samples were reported overall and within each of the sites. For all assays and sample combinations, at least 79 out of 80 replicates gave the correct mutation call. The overall proportion of correct calls was 99.6% (1115/1120); 99.6% (558/560) for mutation-positive (3xLOD) samples and 99.5% (557/560) for wild-type samples.

Proportion of Correct Calls by Assay for Mutation positive and Wild-type samples

Mutant 3X LoD Specimens (Target Control Ct approximately 30)						
12ALA	12ARG	12ASP	12CYS	12SER	12VAL	13ASP
79/80	80/80	80/80	79/80	80/80	80/80	80/80
Wild-Type Specimen						
80/80	79/80	80/80	80/80	79/80	79/80	80/80

Variation (i.e., consistency in calling) was also measured using the GINI index calculated over all laboratories as detailed in the table below. The GINI index measures the variation or consistency in call response. A GINI index of zero (0) means total consistency (i.e., all responses in one single category) while a GINI index of half (0.5) means maximum inconsistency (i.e., the calls are spread equally across the two categories). The results of the GINI analyses when applied to the calls achieved for the 3xLOD and wild-type samples demonstrated very high consistency with the GINI indices over all laboratories less than 0.025 for all assays.

Variance components analyses (random effects models) were used to generate estimates of reproducibility in terms of ΔCt and Ct values. The % CV for the ΔCt and Ct values for each reaction per sample below.

Reproducibility Precision Estimates

	%CV for ΔCt		**%CV for Mutant Ct**		**%CV for Control Ct**		
Assay	**3xLOD**	**C50**	**3xLOD**	**C50**	**3xLOD**	**C50**	**WT**
12ALA	13.14	8.32	1.87	2.02	0.97	1.12	1.12
12ARG	10.79	8.04	1.59	1.96	1.24	1.51	1.15
12ASP	12.86	5.87	1.11	1.00	0.90	0.90	1.04
12CYS	17.61	10.83	1.86	2.02	1.54	1.22	1.15
12SER	13.97	10.43	1.71	2.11	0.94	1.19	1.15
12VAL	9.66	15.47	1.52	1.65	1.11	3.74	1.26
13ASP	13.73	9.35	1.91	2.08	1.11	1.41	1.19

Repeatability Precision Estimates

Assay	%CV for Delta Ct		%CV for Mutant Ct		%CV for Control Ct		
	3xLOD	C50	3xLOD	C50	3xLOD	C50	WT
12ALA	10.71	7.51	1.69	1.76	0.77	0.90	0.79
12ARG	9.83	8.04	1.21	1.76	0.84	1.33	0.90
12ASP	10.16	4.08	0.93	0.89	0.80	0.76	0.76
12CYS	13.15	8.80	1.31	1.76	1.40	1.01	0.76
12SER	6.76	6.18	1.10	1.48	0.80	0.90	0.90
12VAL	9.21	15.32	1.40	1.42	0.91	3.49	0.94
13ASP	8.67	7.01	1.30	1.65	0.91	1.19	0.97

10. <u>Sample Handling Variability Across Three Sites (Extraction Study)</u>

To assess sample handling variability as part of the KRAS Kit test system process, 30 sequential 5-µm sections were cut from each of 10 FFPE CRC samples (3 WT and 1 per mutation). Sections were randomized to one of three testing sites so that each site received 10 sections per FFPE sample (100 sections total). Of the 300 DNA extractions tested, 298 samples were valid. There was 99.3% concordance with respect to the K-Ras mutation calls between the three sites. The variance of ΔCt values for each assay was estimated, and the contribution of between and within laboratory sources was estimated using an ANOVA variance components model. Variance for within-test site was highest for 12ASP (0.30). Variance between-test site was highest for 12SER (0.05). A comparison by site of mean ΔCt values with corresponding SD for mutant and wild-type samples showed very close agreement for results. The results demonstrate the agreement of the DNA extraction procedure and sample processing in conjunction with the KRAS Kit.

Comparison by Site of Mean ΔCt (SD) Values for Mutant Type Samples

	12ALA	12ARG	12ASP	12CYS	12SER	12VAL	13ASP
CLA	2.44 (0.1)	2.62 (0.3)	3.03 (0.6)	2.24 (0.1)	2.34 (0.3)	2.51 (0.1)	3.93 (0.4)
HGX	2.44 (0.2)	2.52 (0.4)	3.01 (0.7)	2.29 (0.2)	2.10 (0.4)	2.44 (0.5)	4.15 (0.7)
MAN	2.67 (0.6)	2.52 (0.2)	3.07 (0.5)	2.29 (0.2)	2.74 (0.5)	2.56 (0.2)	3.95 (0.3)

Comparison by Site of Mean ΔCt (SD) Values for Wild-Type Samples

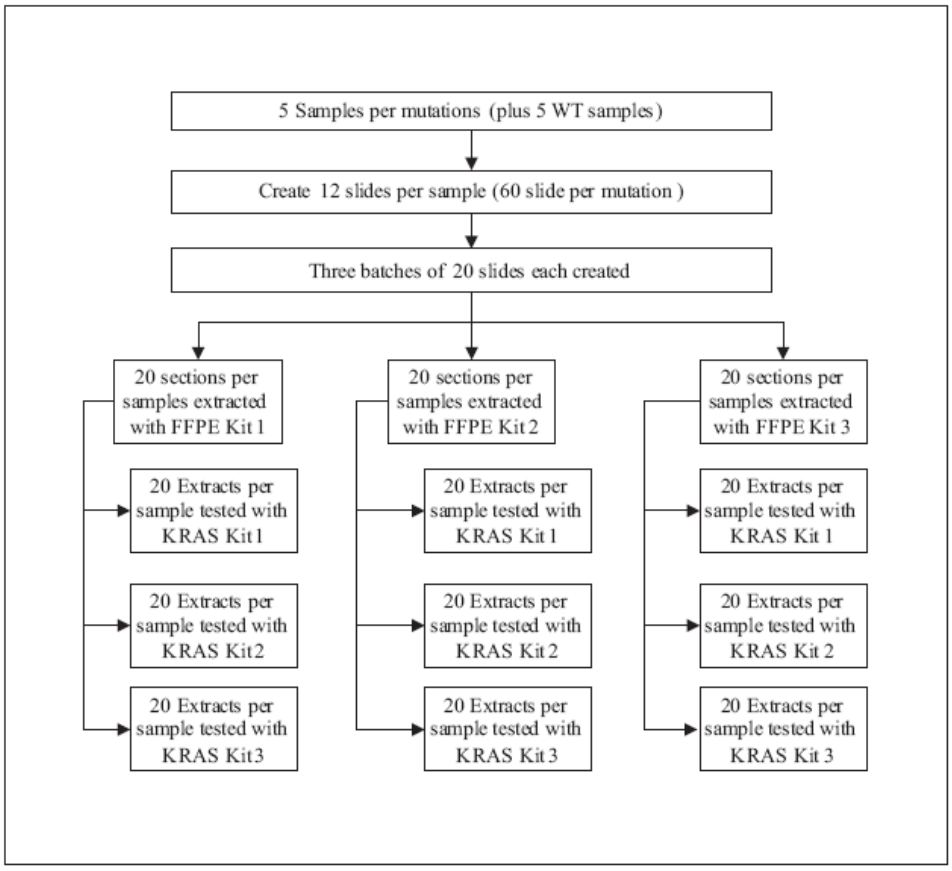
	12ALA	12ARG	12ASP	12CYS	12SER	12VAL	13ASP
CLA	12.46 (0.3)	-	10.37 (0.4)	-	11.84 (0.4)	12.36 (0.5)	11.11 (0.6)
HGX	12.09 (0.6)	13.07 (0.2)	10.17 (0.5)	-	11.71 (0.7)	12.20 (0.6)	11.00 (0.9)
MAN	12.07 (0.2)	-	10.61 (0.4)	-	11.94 (0.3)	12.28 (0.6)	11.82 (0.5)
Note: "-" illustrates a missing value, due to no break through being observed							

11. Specimen Handling – Minimum Tumor content and Macrodissection

To support the consistent mutation calling in macrodissected CRC samples with tumor proportion ≤ 20%, DNA extracted from thirteen samples whose tumor proportion ranged from 3% to 18% were either macrodissected or not. The samples were tested in duplicate. The overall accuracy of the macrodissected samples was compared to the non-macrodissected counterparts as well as ten samples whose tumor proportion was > 20% but close to the cut-off (range 21% to 30%). All results were compared to bi-directional sequencing which were also macrodissected to enrich for tumor. The KRAS Kit did not incorrectly detect a mutation in six (6) samples identified as wild-type by bi-directional sequencing in either the macrodissected and non-macrodissected sections. One (1) wild-type sample produced invalid results with the KRAS Kit across all replicates. Four (4) samples identified as mutant by bi-directional sequencing were also identified as mutant by the KRAS Kit. One (1) mutant positive sample by bi-directional sequencing was correctly detected by the KRAS Kit when the sample was macrodissected but not when the sample was not macrodissected, supporting the use of macrodissection. One sample produced inconclusive results by bi-directional sequencing. The results support the ability of the KRAS Kit to detect mutant in samples with low percentage tumor when the specimen is macrodissected. Additionally, sections from the specimens used in the correlation to bi-directional sequencing studies had tumor proportion (% tumor content) that spanned the range of approximately 10% to 99%. The accuracy results support the use of samples whose tumor proportion is greater than 20% without macrodissection.

12. Lot-to-Lot Reproducibility

The potential for lot-to-lot variability to impact the mutation detection was assessed. In this study, three lots of QIAamp® DSP DNA FFPE Tissue Kit (FFPE Extraction Kit) and *therascreen*® KRAS RGQ PCR Kit with each lot of FFPE Extraction were evaluated. An overview of the workflow for this study is shown below:



For this study, five FFPE CRC specimens (FFPE CRC) for six of the seven mutations, plus five wild-type (WT) samples, were obtained. Only two FFPE CRC specimens were available for the 12ARG mutation, so these studies were supplemented with one 12ARG FFPE cell line. For each mutant sample (except for the 12ARG mutation) and the WT Sample, twelve sequential 5-µm sections were cut and mounted onto glass slides. For the 12ARG mutation, twenty-four sections were cut from each of the two 12ARG FFPE Samples and twelve sections were cut from the FFPE Cell Line for 12ARG. Thus a total of 60 sections per mutation and WT were obtained. These 60 sections were randomly assigned to one of three batches, to give three batches of twenty slides per mutation and WT. DNA was extracted and each extracted DNA sample was tested using the control reaction and its corresponding mutation reaction. Each DNA extract was tested singly on three separate KRAS Kit runs. The acceptance criterion for this study was that for each of the mutation assays and the three FFPE Kit Lots tested, the proportion of correct mutation calls would be at least 59/60. All samples met the acceptance criteria with the exception of 12SER and 12VAL, the latter of which was found to be due to reasons unrelated to the kits. To ensure quality across lots, a supplemental study was conducted using FFPE cell lines (one wild type and 7 mutant samples were extracted with 3 lots of FFPE Extraction Kit to yield DNA samples with target control CT values that span the range of total DNA input level for the KRAS Kit. The extracted DNA samples were then tested against 3 lots of KRAS Kit using the strategy outlined above.

The mutation status for all samples tested with the different FFPE extractions kit lots and KRAS Kit lots was 100% correct. For the mutant samples, the respective ΔCt value and the corresponding mutation status were calculated. The mutation status for all samples tested with the different FFPE extractions kit lots and KRAS Kit lots was 100% correct. This study criteria was met and the results demonstrated that there is no significant variability between lots of QIAamp® DSP DNA FFPE Tissue kit (FFPE extraction kit) and lots of *therascreen*® KRAS RGQ PCR Kit.

13. Guard Band Studies

The potential impact of using different proteinase K digestion times during the DNA extraction process as well as altering the RGQ PCR cycling parameters for the KRAS Kit were investigated. Changes were assessed for any potential effects on mutation reporting. The following studies were conducted to assess the robustness of the Extraction Kit and the KRAS Kit:

Proteinase K Digestion Times: FFPE CRC specimens were used in the evaluation of different proteinase K digestion times on the potential to impact mutation calling using the KRAS Kit. The current proposed labeling for the KRAS Kit states that proteinase K digestion of samples should be carried out for 60 minutes. Therefore, in order to assess the robustness of this limit, FFPE samples representing the wild type and each of the 7 seven K-Ras mutations, detected by the KRAS Kit, were extracted using digestion times that bracket the stated time. Five time points were tested in total; 50, 55, 60, 65, and 70 minutes respectively. Six replicate extractions were carried out at each time interval. A total of 240 extractions were carried out (6 replicates x 5 time intervals x 8 FFPE CRC Samples). Across all assays, there was 1 replicate of 240 that resulted in a false negative at the 65 minute time point. The KRAS Kit PCR is robust to varying the Proteinase K digestion step by ±10 minutes, from the stated 60 minute digestion.

RGQ PCR Cycling Analysis: Denaturing and annealing temperatures were investigated. Denaturing temperatures are required for the complete separation of target DNA strands and annealing temperature is required for the specific binding of primers prior to extension. The KRAS Kit cycling uses a denaturing temperature of 95°C and an annealing temperature of 60°C. The combined effect of altering each of these parameters was tested in 9 combinations (inclusive of the standard conditions). These combinations are presented in Table below. The KRAS Kit PCR is robust to varying the melting and annealing temperatures of up to ±1°C (in any combination). All mutation calls for each of the FFPE samples tested at 9 different combinations of cycling conditions returned a 100% correct mutation status. Changes in the cycling temperatures had no impact on the results of mutation status.

Guard Band PCR Cycling Conditions

		Melting Temperature		
		59°C	60°C	61°C
Denaturing Temperature	94°C	94°C /59°C	94°C/60°C	94°C/61°C
	95°C	95°C/59°C	95°C/60°C	95°C/61°C
	96°C	96°C/59°C	96°C/60°C	96°C/61°C

PCR Set-up and Stability Times: The goal of the study was to determine the robustness of the *therascreen* KRAS RGQ PCR Kit to different temperatures for different periods of time before it the reactions are loaded on the MDx Instrument. Three parameters were tested in the study:

1. Stability of reaction mixes/Taq polymerase and Positive Control was assessed to determine the effect of time on the stability of reaction mixtures at room temperature in the event that some experiments take longer to set up than the stipulated 1 hour. The following times were tested: (1) 1 hour at room temperature, (2) 4.5 hours at room temperature or (3) 6 hours at room temperature.
2. The temperature of storage of the Rotor-Gene® Q (RGQ) tubes between start of PCR set up and start of the RGQ run was assessed to determine the effect of temperature on the stability of the reaction mixes/Taq with the DNA sample. Room temperature and 2-8°C were tested.
3. The time between start of PCR set up and start of the RGQ run was assessed to determine the stability of the reaction mixes/Taq with the DNA sample added. Tubes were kept for 1 hour, 2 hours, 7 hours or overnight (18 hours).

The following samples were tested:

(1) Positive Control - Sample guaranteed to give a Ct value within the acceptable range.

(2) Wild type and 3X LOD samples - Samples aimed to challenge the assay (i.e., low positive Control).

(3) No Template control - Negative Samples

The impact of time and temperature on the performance of the KRAS Kit was assessed by comparing the Ct values generated for all samples across 11 experimental conditions (interchangeable times and temperatures described above). Each known mutation sample was tested with its appropriate reaction mix while the WT sample was tested with the Control reaction mix. Each assay was run in triplicate. The data demonstrates that pre- and post-setup, the KRAS Kit can be stored on the bench-top or at 4°C for sufficient periods of time to allow

customers flexibility in the working day. The times within which the KRAS Kit can be stored both for pre- and post-setup are detailed in the table below.

Times within which the KRAS Kit can be stored for pre- and post-setup

Thaw Time		Storage Temp after PCR setup	PCR Setup and Storage time
Minimum	Maximum		
1 hour	**4.5 hours**	**Room Temperature**	**7 hours**
1 hour	**4.5 hours**	**2-8°C**	**18 hours**

Note: PCR setup is to be performed at room temperature. 'Storage' refers to the time between completion of PCR setup and start of the PCR run on the RGQ platform.

14. Cross-Contamination

Studies were performed to demonstrate the absence of cross-contamination between test samples. Two FFPE cell lines were used for this study: one containing WT cells only, and the other containing cells harboring the 12ALA mutation. The 12ALA mutation was selected for this study because the 12ALA reaction is the most sensitive reaction in the KRAS Kit as determined in the analytical sensitivity studies, and therefore most prone to exhibit false positive results from contamination. Multiple serial 5-µm sections were prepared and mounted onto glass slides for testing. DNA was extracted and assayed using one lot of reagents and one RGQ instrument according to protocol. Each extract was tested in 7 replicates using input concentrations based on control Ct in the midrange (approximating Ct 26). The study consisted of ten test runs designed to investigate the potential for contamination both within and between runs. The 10 runs were divided between two sets (5 runs per set) where the sequence in which reagents are added to reaction wells differed; for set "a" the No Template Control preceded the positive control according to instructions for use, and for set "b" the No Template Control preceded the test sample to maximize the opportunity to detect cross-contamination. Results were summarized by Ct, ΔCt and call. The results demonstrated one invalid result from a WT sample replicate. No false positive results were detected. No false positive results were detected. The results of this study indicate no detectable contamination.

15. Stability-Specimen

a) Clinical Specimen, Slide-Mounted

To assess the stability of slides prepared from FFPE CRC tissue samples to determine limits of suitability for the KRAS Kit, ten 5-µm sections were cut from each of eight FFPE CRC specimens (one for each mutation and one wild-type), mounted onto glass slides, and stored in the dark at room temperature for four weeks. The acceptance criteria were that at each time point, the mutation status must agree with that determined at initial baseline testing, and for each time point the change in ΔCt relative to the corresponding baseline time point must not be statistically different, or the

upper 95% CI for the absolute mean change ΔCt must be less than 1. For each time point tested, DNA was extracted from 2 slides, pooled and tested in 5 replicates on Day 7, Day 14 and Day 28. The wild-type slides met the stability criteria across all time points based upon consistent mutation status. Slides from 4 of the 7 mutations (12ALA, 12CYS, 12SER, and 12VAL) were demonstrated to meet stability criteria across all 4 time points. Slides from the 12ASP and 12ARG mutations met all acceptance criteria for all time points where valid results were obtained. No trend in the values was observed for the FFPE samples tested. The results of this study overall support the claim that slides prepared from FFPE samples can be stored for up to 4 weeks at room temperature in the dark prior to testing with the KRAS Kit.

b) Extracted Clinical Specimen DNA

To assess the stability of DNA extracted from FFPE CRC samples (representing 7 mutations and 1 wild-type) using the QIAamp® DSP FFPE Tissue Kit, samples were stores at 2°C to 8°C for 7 days followed by storage at -18°C to -22°C for 5 weeks. During storage at -18°C to -22°C, the samples were subjected to multiple freeze-thaw cycles. DNA extracts were freeze/ thawed over 2 hours at room temperature and then returned to freezer. DNA extracts were tested on days 14, 21, and 35. Five test replicates of all DNA extracts at each time point were evaluated. The acceptance criteria were that the upper limit of the 95% CI cannot exceed the baseline value by more than 1ΔCt). Determination of mutation status was demonstrated to be consistent under the conditions of the study. There was 1 replicate that resulted in false positive call that was attributed to human error. The acceptance criteria were met. DNA extracted from FFPE samples is stable and suitable for use with the KRAS Kit when stored for up to 7 days at 4°C with additional storage at -20°C up to 5 weeks with multiple freeze thaws.

16. Stability-Reagents

The Stability studies conducted for both the *therascreen*® KRAS RGQ PCR Kit and the QIAamp DSP DNA Extraction Kit include (1) Real-time storage conditions for closed bottle conditions, open bottle, and open bottle simulating multiple time use by the user for both the KRAS Kit and the Extraction Kit; (2) Transport conditions (extreme temperatures during storage and shipping, including inversion of reagents), and (3) Stress (freeze/thaw) conditions. FFPE CRC clinical samples and FFPE cell lines were used for this investigation. DNA was extracted according to protocol and each mutation DNA extract was normalized with wild-type DNA to provide test samples corresponding to 3x and 9x the LoD of each of the 7 mutation reactions. Testing was conducted in triplicate with the exception of the open-bottle (multi-use studies) which is run in singlicate. The acceptance criteria for each time point are that (1) the correct call is made, and (2) the ΔCt values of each sample, when plotted against time, do not indicate a statistically significant trend following regression analysis. Testing was conducted at 4 months and is planned to continue to 36 months for the KRAS Kit

and 24 months for the Extraction Kit. Freeze thaw studies up to 4 months were conducted 12 times. The data supports the following stability claims:

- Storage and shelf life for the KRAS Kit is 4 months at-20°C±5°C
- When used with this assay and the modified protocol, storage and shelf life for the QIAamp kit is 4 months at ambient temperature at 15°C to 25°C except for QIAamp MinElute Columns (5°C ±3°C).

Overview of the Stability Studies and Temperatures

Study*	Storage condition		Cycling condition	
	definition	temperature	definition	temperature
Closed bottle	Real time	-20°C±5°C	n.a.	n.a.
Open bottle	Real time	-20°C±5°C	n.a.	n.a.
Open bottle (light sensitivity)	Ambient temperature	22°C±3°C (Incubator)	n.a.	n.a.
Transport simulation study KRAS kit	Real time	-20°C±5°C	Extreme cold	-90°C to -65°C
			Interim storage	-20°C±5°C
			Thawing	22°C±3°C***
			Freezing	-20°C±5°C
Transport simulation study QIAamp kit	Real time	25°C±3°C (incubator)	Extreme cold	-20°C±5°C
			Extreme warm	45°C±3°C
Stress test	Real time	-20°C±5°C	Thawing	22°C±5°C***
			Freezing	-20°C±5°C

* QIAamp kit stored at (25°C±3°C, incubator), QIAamp MinElute Columns at (5°C±3°C). The cycling for the transport simulation studies performed with all kit components including the QIAamp MinElute columns. *** Tubes are inverted.

Stability- Open bottle study (light sensitivity/ reaction mix + enzyme stability): Master mixes were prepared (Reaction mix + enzyme) in clear tubes and used at predefined times following preparation up to 3 hours. The results indicate that KRAS Kit Master Mix is stable for at least two hours when stored at 32°C.

B. Animal Studies

None.

C. Additional Studies

None.

X. SUMMARY OF PRIMARY CLINICAL STUDIES

A clinical performance study was conducted to generate data to support the clinical utility of the *therascreen*® KRAS RGQ PCR Kit (referred to as KRAS Kit) as a companion diagnostic test that aids in the identification of patients for treatment with cetuximab (Erbitux®). The objective of the study was to assess whether K-Ras status as determined by the *therascreen* KRAS RGQ PCR Kit can be used to select patients with metastatic colorectal cancer (mCRC) who will benefit from cetuximab treatment.

CA225025 (ClinicalTrials.gov number NCT00079066) was a randomized, multicenter, open-label, Phase 3 study of cetuximab combined with best supportive care (BSC) versus BSC alone in patients with previously treated, epidermal growth factor receptor (EGFR) - expressing, recurrent or metastatic colorectal cancer (mCRC). The study was conducted by the National Cancer Institute of Canada Clinical Trials Group (NCIC CTG).

Banked tumor samples from patients in study CA225025 were tested with the KRAS Kit to identify two subgroups: K-Ras mutation-positive and K-Ras mutation-negative (wild-type), according to whether at least one or none of seven K-Ras mutations in codons 12 and 13 of exon 2 in the K-Ras oncogene was detected. In retrospective analyses, efficacy data from study CA225025 were stratified by K-Ras subgroup.

Note: The KRAS Kit is designed to specifically detect 7 K-Ras mutations in codon 12 and 13 of the K-Ras gene. It is not designed to specifically detect the wild-type sequence at these codons. The results of the test are reported out as "Mutation-positive" and "No mutation detected." Generally, discussions of patient response to cetuximab therapy in the context of K-Ras status have referred to two groups; K-Ras wild-type and K-Ras mutant. In the report that follows, the *therascreen* "no mutation detected" result is referred to as K-Ras mutation-negative (wild-type) to be consistent with the pharmaceutical manufacturer's designations in the cetuximab product label. Patients in the clinical study who were K-Ras mutation-positive tested positive for one or more of the 7 mutations detected by the *therascreen* KRAS RGQ PCR Kit (G12A, G12D, G12R, G12C, G12S, G12V, G13D). Patients in the clinical study who were K-Ras mutation-negative (wild-type) tested negative for the 7 mutations detected by the *therascreen* KRAS Kit. However they may have harbored mutations in the K-Ras gene not identified by the KRAS Kit such as 13CYS, or elsewhere in the gene such as codon 61).

The data presented in this PMA support the clinical utility of the *therascreen* KRAS RGQ PCR Kit and support corresponding changes to the cetuximab (Erbitux®) labeling for the mCRC indication. The results demonstrated that the efficacy of cetuximab in prolonging overall survival (OS) and progression-free survival (PFS) was statistically significant in patients with K-Ras mutation-negative (wild-type) status. In patients with K-Ras mutation-positive tumors, no statistically significant difference was observed between the two treatment groups in OS or PFS.

A. Study Design

1. *CA225025 Trial*

CA225025 was initiated on August 28, 2003 and closed to randomization on August 26, 2005 after 572 patients were randomized to either cetuximab + BSC or BSC alone. Randomization was stratified by center and Eastern Cooperative Oncology Group (ECOG) performance status (PS) (0 or 1 vs. 2). The study was open label, i.e., patients and investigators were not blinded to treatment assignment. Investigators at 30 centers in Canada and 28 centers in Australia, New Zealand, and Singapore enrolled at least 1 patient. Cetuximab was administered on a weekly dosing schedule until disease progression or until other conditions including unacceptable toxicity, symptomatic disease progression, and need for standard radiation treatment for index lesions, led to discontinuation from protocol treatment. The study was completed on March 6, 2006. An application to the FDA (CDER) in support of this indication was approved on October 2, 2007 (BLA 125084). The study demonstrated a clinically relevant and highly significant improvement in overall survival (OS), as well as longer progression-free survival (PFS) and higher overall response rates.

Assessment of K-Ras mutation status was not prospectively planned in the original protocol for the CA225025 study because evidence for the impact of K-Ras mutation on therapeutic response was unavailable at the time. However, based on emerging data demonstrating K-Ras mutation status as a potential predictive biomarker for EGFR-targeted monoclonal antibody therapies, available tumor samples from patients in the CA225025 study were tested using bi-directional sequencing to investigate if K-Ras status predicted patient response to cetuximab treatment (1). The results are the basis for the development of planned, retrospective analyses evaluating the influence of K-Ras mutation status on OS and PFS with a specific KRAS companion diagnostic (i.e., the KRAS Kit).

2. *Patients*

Patients were males and females who were at least 16 years of age and had EGFR positive colorectal cancer tissue and an ECOG PS of 0 to 2. All patients were individuals who had failed all available chemotherapeutic agents, including an irinotecan-containing regimen and an oxaliplatin-containing regimen, for treatment of metastatic disease, and for whom no standard anticancer therapy was available. The only remaining standard available therapy as recommended by the investigator was BSC.

3. *Tumor Specimens and Testing*

Tumor tissue blocks and sections were collected prior to enrollment in CA225025. The median age of sample at testing was close to 8.5 years. Tumor samples were mostly from primary tumor specimens (~90%). However, a small number of samples were derived from metastatic lesions. The average tumor content was close to 50% and less than 10% of the samples required macrodissection. Most patients (~81%) had necrosis scored on the tumor sample.

Tissue reserves were in the form of formalin-fixed paraffin embedded (FFPE) blocks or unstained slides containing sections from FFPE blocks. HistoGeneX laboratories (Antwerp, Belgium) performed the K-Ras evaluations using the KRAS Kit without knowledge of the subject's site, identification number, or clinical outcome, including tumor response. For all CA225025 subjects with sufficient tissue reserves, 5-micron sections adhered on glass slides underwent fresh hematoxylin and eosin (H&E) staining and pathologist verification to make determinations regarding necrosis and tumor content in sample prior to K-Ras assessment using the KRAS Kit. When tumor content was deemed $\leq 20\%$ in H&E stained sections, the pathologist marked tumor borders to facilitate macro-dissection of tumor tissue from 1 or more 5-micron sections for a total area of ≥ 4 mm^2 tumor in accordance with the KRAS Kit labeling recommendations.

Descriptive information about the tumor samples was collected and summarized across the treatment arms and by K-Ras status for the purpose of evaluating the potential for bias in the analyses. A summary of the information is provided below.

	KRAS Wild-type		KRAS Mutant	
	C+BSC N = 117	BSC N = 128	C+BSC N = 108	BSC N = 100
Age of sample at testing, years				
N	104	107	93	88
Median	8.7	8.3	8.4	8.4
Min - Max	6.7 - 15.8	6.5 - 14.6	6.1 - 14.2	6.7 - 14.9
Disease Stage, n(%)				
TNM Classification	42 (35.9)	40 (31.3)	32 (29.6)	30 (30.0)
CRC stage	0	0	1 (0.9)	0
Stage IV	0	0	1 (0.9)	0
Duke's stage	17 (14.5)	20 (15.6)	11 (10.2)	11 (11.0)
A	1 (0.9)	0	0	0
B	4 (3.4)	6 (4.7)	1 (0.9)	5 (5.0)
C	10 (8.5)	13 (10.2)	9 (8.3)	6 (6.0)
D	2 (1.7)	1 (0.8)	1 (0.9)	0
Missing disease stage	58 (49.6)	68 (53.1)	64 (59.3)	59 (59.0)
Tumor type, n (%)				
Primary	106 (90.6)	116 (90.6)	97 (89.8)	91 (91.0)
Left Colon/Rectum	83 (70.9)	88 (68.8)	58 (53.7)	55 (55.0)
Right Colon	13 (11.1)	15 (11.7)	31 (28.7)	28 (28.0)
Transverse Colon	6 (5.1)	6 (4.7)	8 (7.4)	3 (3.0)
Missing	0	2 (1.6)	0	0
Other	4 (3.4)	5 (3.9)	0	5 (5.0)
Metastatic	10 (8.5)	11 (8.6)	11 (10.2)	6 (6.0)
Liver	5 (4.3)	5 (3.9)	3 (2.8)	4 (4.0)
Lung	0	2 (1.6)	1 (0.9)	0
Lymph Node	4 (3.4)	3 (2.3)	3 (2.8)	1 (1.0)
Other	1 (0.9)	1 (0.8)	4 (3.7)	1 (1.0)
Missing	1 (0.9)	1 (0.8)	0	3 (3.0)
Sampling method, n (%)				
Biopsy	12 (10.3)	27 (21.1)	15 (13.9)	15 (15.0)
Resection	100 (85.5)	99 (77.3)	93 (86.1)	81 (81.0)
Missing	5 (4.3)	2 (1.6)	0	4 (4.0)
Area of sample tissue, mm**2				
N	117	128	108	100
Median	315.6	305.8	325.2	294.4
Min - Max	8.7 - 598.9	3.2 - 679.1	4.9 - 595.7	3.6 - 603.9
Area of tumor tissue, mm**2				
N	117	128	108	100
Median	131.8	124.5	130.5	133.7
Min - Max	4.0 - 474.1	1.7 - 611.3	2.1 - 378.7	1.4 - 335.7
Tumor content in sample, %				
N	117	128	108	100
Median	47.0	47.5	45.0	51.0
Min - Max	8.0 - 100.0	7.0 - 100.0	5.0 - 100.0	1.0 - 100.0
Macro-dissection of sample, n(%)				
Yes	10 (8.5)	12 (9.4)	12 (11.1)	5 (5.0)
No	107 (91.5)	116 (90.6)	96 (88.9)	95 (95.0)

Necrosis score in tumor area, n (%)				
No necrosis	18 (15.4)	25 (19.5)	17 (15.7)	24 (24.0)
<10% necrosis	73 (62.4)	78 (60.9)	57 (52.8)	54 (54.0)
10-50% necrosis	22 (18.8)	19 (14.8)	28 (25.9)	17 (17.0)
>50% necrosis	4 (3.4)	6 (4.7)	6 (5.6)	5 (5.0)
Necrosis score outside of tumor area, n (%)				
No necrosis	107 (91.5)	123 (96.1)	103 (95.4)	94 (94.0)
<10% necrosis	8 (6.8)	4 (3.1)	4 (3.7)	4 (4.0)
10-50% necrosis	1 (0.9)	1 (0.8)	1 (0.9)	1 (1.0)
>50% necrosis	1 (0.9)	0	0	1 (1.0)

CRC=colorectal cancer; TNM=tumor node metastasis

4. *Clinical Inclusion and Exclusion Criteria*

a. Inclusion and Exclusion Criteria for Specimen Testing:
Specimens had to have sufficient tissue sample for testing, H & E assessment, and appropriate informed consent.

b. Inclusion and Exclusion Criteria for Patient Enrollment into CA225025 Trial
A summary of the pertinent criteria for enrollment into the trial was limited to patients who met the following inclusion and exclusion criteria:

Inclusion criteria:

- EGFR positivity of representative samples of diagnostic tumor tissue by immunohistochemistry, performed by a reference laboratory.
- Received a prior thymidylate synthase inhibitor for adjuvant and/or metastatic disease, which may have been given in combination with oxaliplatin or irinotecan.
- Received and failed an irinotecan (CPT-11) –containing regimen (single agent or in combination) for treatment of metastatic disease, OR relapse within 6 months of completion of an irinotecan-containing regimen.
- Received and failed an oxaliplatin –containing regimen (single agent or in combination) for treatment of metastatic disease, OR have documented unsuitability for an oxaliplatin- containing regimen
- Measurable or evaluable disease
- The only remaining standard available therapy as recommended by the Investigator was best supportive care.
- Adequately recovered from recent surgery, chemotherapy, and/or radiation therapy. At least 4 weeks must have elapsed from major surgery, or treatment.
- ECOG performance status of 0, 1, or 2
- Imaging investigations including chest x-ray and CT/MRI of abdomen/pelvis or other scans as necessary to document all sites of disease done within 28 days prior to randomization.
- ECG done within 28 days prior to randomization

- Hematology and Biochemistry done within 14 days prior to randomization and with initial values within specified ranges.
- Age >16 years
- Women of child bearing potential must have a negative serum or urine pregnancy test within 72 hours prior to randomization
- Patient consent
- No concurrent enrollment in a clinical study

Exclusion criteria:

- History of other malignancies
- Women who are pregnant or breastfeeding.
- Any active pathological condition which would render the protocol treatment dangerous or impair the ability of the patient to receive protocol therapy.
- Any condition that would not permit compliance with the protocol
- History of uncontrolled angina, arrhytmias, cardiomyopathy, congestive heart failure, or documented myocardial infarction within the 6 months preceding registration
- Symptomatic metastases in the central nervous system
- Prior cetuximab or other therapy which targets the EGFR pathway
- Prior murine monoclonal antibody therapy
- Severe restrictive lung disease or radiological pulmonary findings of interstitial lung disease on the baseline chest x-ray
- Receipt of an a experimental therapeutic agent within the past 30 days

5. *Follow-up Schedule*

Patients were monitored according to protocol during the prospective trial.

6. *Clinical Endpoints*

Regarding safety, the review of adverse events occurred during the BLA review by the lead clinical reviewer. The BLA was approved in October 2007. No new safety issues were raised with the planned, retrospective analysis of samples from the clinical trial to evaluate effects of KRAS kit mutation status on cetuximab efficacy. Refer to the section titled Safety Results below for information about safety based on K-Ras subgroups.

Regarding efficacy, the primary endpoint in the original protocol for the CA225025 study was overall survival (OS). A secondary endpoint was progression-free survival (PFS).

The primary objective of the planned, retrospective analysis was to support the clinical utility of the *therascreen*® KRAS RGQ PCR Kit as an aid in identifying patients with metastatic CRC for treatment with cetuximab based on OS and PFS efficacy data. Efficacy was stratified by K-Ras subgroup (mutation-positive or mutation-negative). The predictive effect of K-Ras mutation status on cetuximab

OS and PFS was evaluated with a test for the interaction between treatment group and K-Ras mutation status using a stratified Cox proportional hazards model that included treatment group, K-Ras mutation status, and the interaction between the two as factors. Sensitivity analyses were performed to assess the potential impact of missing K-Ras evaluations on OS.

B. Accountability of PMA Cohort

The patients used for analysis populations were:

- All Randomized Patients: all enrolled patients who were randomized
- All Treated Patients: all randomized patients who received at least one dose of medication

The following subsets were created based on the KRAS Kit test result:

- *K-Ras Evaluated*: All patients who had K-Ras status available
- *K-Ras Mutation-negative (wild-type)*: K-Ras Evaluated who had K-Ras no-mutation detected tumors (negative for the 7 mutations in codon 12 and 13 detected by the kit)
- *K-Ras Mutation-positive*: K-Ras Evaluated who had K-Ras mutation-positive tumors (positive for one or more of the 7 mutations in codon 12 and 13 detected by the Kit)
- *K-Ras Not Evaluated*: All patients who had no K-Ras status available

A total of 572 patients were randomized (287 cetuximab + BSC, 285 BSC) over a period of 2 years (August 28, 2003 to August 26,-2005) in 30 centers in Canada, and 28 centers in Australia, New Zealand, and Singapore. Randomization was by center and ECOG PS. No notable imbalances in the proportion of patients randomized at each center within the K-Ras Evaluated and K-Ras Not Evaluated populations, and within K-Ras mutation-negative (wild-type) and K-Ras mutation-positive subsets were observed. Patient disposition of the K-Ras Evaluated and the K-Ras Not Evaluated Set across treatment arms is shown below. The K-Ras Evaluated population represented 79.2% (453/572) of the All Randomized population. The K-Ras mutation-negative (wild-type) subset represented 54.1% (245/453) and the K-Ras mutation-positive subset represented 45.9% of the K-Ras Evaluated population.

Summary of All Randomized Patients Analysis Populations by Availability for KRAS Kit Testing

	All Randomized		K-Ras Evaluated		K-Ras Not Evaluated		K-Ras mutation-negative (wild-type)		K-Ras mutation-positive	
	Cet + BSC	BSC	Cet + BSC	BSC	Cet + BSC	BSC	Cet + BSC	BSC	Cet + BSC	BSC
Randomized	287	285	225	228	62	57	117	128	108	100
Treated	288	274	228	218	60	56	118	124	110	94
Never Treated	4	6	2	5	2	1	1	2	1	3

BSC = best supportive care; Cet + cetuximab; 5 subjects were randomized to BSC and received cetuximab + BSC.

Tumor samples for K-Ras testing were available for 84.4% (483/572) of the patients in the trial; 84.3% (242/287) in the treatment arm and 84.6% (241/285) in the control arm. There were no imbalances between treatment groups in the proportion of patients with missing tumor samples (15.7% vs. 15.4%). The most common reason for missing tumor samples was lack of informed consent. Of the 483 subjects with tumor tissue available, there were an additional 27 samples unavailable for K-Ras retesting with the KRAS Kit for this study. The final number of patients with evaluable tumor was 453/572 or 79.2% (K-Ras Evaluated Population) of which 78.4% of patients were in the treatment arm and 80.0% of patients were in the control arm. Reasons for missing tumor tissue samples and lack of results are summarized in the table below:

Reasons for Missing Tumor Samples for KRAS Kit Testing

All Randomized Subjects		
	Number of Subjects	
	Cetuximab + BSC (N=287)	BSC (N= 285)
Subjects with tumor tissue samples	242	241
Subjects without tumor tissue samples	45	44
Reasons for missing tumor tissue samples:		
Subject died before consent	16	15
Subject not consented	15	11
Insufficient tissue available	7	8
Site not participated	4	4
Lack of informed consent/lost to follow-up	2	5
Tissue could not be released for K-Ras testing	1	1
Reasons for Missing K-Ras evaluation:		
Failure at H& E assessment step:	13	10
Absence/insufficient tumor cells in sample	8	8
Incorrect tumor type (tubular adenoma lacking malignant cells)	4	2
Unacceptable tissue sample	1	0
Failure to obtain enough DNA from sample	0	0
Failure to obtain an assessment using the test kit/invalid	4	3
TOTAL K-Ras EVALUABLE SET (n= 453)	225	228

C. Study Population Demographics and Baseline Parameters

Demographics

The demographics of the study population are similar to that of the US. The patient demographic characteristics were gender, race, age, ECOG performance status and body surface area. The distributions of demographic variables for all 572 randomized patients in the trial are shown below.

Demographic Characteristics – All Randomized Patients, K-Ras Evaluated, and K-Ras Not Evaluated

	All Randomized Subjects		*K-Ras* Evaluated		*K-Ras* Not Evaluated	
	C+BSC N = 287	BSC N = 285	C+BSC N = 225	BSC N = 228	C+BSC N = 62	BSC N = 57
Gender, n (%)						
Female	101 (35.2)	103 (36.1)	73 (32.4)	80 (35.1)	28 (45.2)	23 (40.4)
Male	186 (64.8)	182 (63.9)	152 (67.6)	148 (64.9)	34 (54.8)	34 (59.6)
Race, n (%)						
White	258 (89.9)	250 (87.7)	207 (92.0)	207 (90.8)	51 (82.3)	43 (75.4)
Black of African or Caribbean Heritage	5 (1.7)	4 (1.4)	3 (1.3)	3 (1.3)	2 (3.2)	1 (1.8)
Black/Asian	0	1 (0.4)	0	1 (0.4)	0	0
Asian	20 (7.0)	25 (8.8)	12 (5.3)	12 (5.3)	8 (12.9)	13 (22.8)
American Indian or Alaska Native	0	2 (0.7)	0	2 (0.9)	0	0
Unknown	4(1.4)	3 (1.1)	3 (1.3)	3 (1.3)	1 (1.6)	0
Age (years)						
N	287	285	225	228	62	57
Median	63.0	63.6	63.0	63.4	62.6	64.8
Min - Max	28.6 - 88.1	28.7 - 85.9	28.6 - 88.1	28.7 - 85.9	29.9 - 78.5	40.0 - 85.8
Age Group, n (%)						
<65	177 (61.7)	158 (55.4)	136 (60.4)	129 (56.6)	41 (66.1)	29 (50.9)
>=65	110 (38.3)	127 (44.6)	89 (39.6)	99 (43.4)	21 (33.9)	28 (49.1)
ECOG Performance Status, n (%)						
0	72 (25.1)	64 (22.5)	61 (27.1)	49 (21.5)	11 (17.7)	15 (26.3)
1	148 (51.6)	154 (54.0)	118 (52.4)	127 (55.7)	30 (48.4)	27 (47.4)
2	67 (23.3)	67 (23.5)	46 (20.4)	52 (22.8)	21 (33.9)	15 (26.3)
BSA (m**2)						
N	287	285	225	228	62	57
Median	1.8	1.8	1.8	1.8	1.8	1.8
Min - Max	1.3 - 2.5	1.3 - 2.5	1.4 - 2.5	1.3 - 2.4	1.3 - 2.3	1.4 - 2.5

BSA = body surface area; ECOG = Eastern Cooperative Oncology Group

Demographic Characteristics – All Randomized Patients by K-Ras Mutation Status

	KRAS Wild-type		KRAS Mutant	
	C+BSC N = 117	BSC N = 128	C+BSC N = 108	BSC N = 100
Gender, n (%)				
Female	33 (28.2)	42 (32.8)	40 (37.0)	38 (38.0)
Male	84 (71.8)	86 (67.2)	68 (63.0)	62 (62.0)
Race, n (%)				
White	108 (92.3)	117 (91.4)	99 (91.7)	90 (90.0)
Black of African or Caribbean Heritage	1 (0.9)	1 (0.8)	2 (1.9)	2 (2.0)
Black/Asian	0	1 (0.8)	0	0
Asian	7 (6.0)	8 (6.3)	5 (4.6)	4 (4.0)
American Indian or Alaskan Native	0	0	0	2 (2.0)
Unknown	1 (0.9)	1 (0.8)	2 (1.9)	2 (2.0)
Age (years)				
N	117	128	108	100
Median	63.8	64.1	61.3	61.8
Min - Max	28.6 - 88.1	35.5 - 85.2	33.6 - 85.9	28.7 - 85.9
Age Group, n (%)				
<65	68 (58.1)	68 (53.1)	68 (63.0)	61 (61.0)
>=65	49 (41.9)	60 (46.9)	40 (37.0)	39 (39.0)
ECOG Performance Status, n (%)				
0	35 (29.9)	25 (19.5)	26 (24.1)	24 (24.0)
1	58 (49.6)	72 (56.3)	60 (55.6)	55 (55.0)
2	24 (20.5)	31 (24.2)	22 (20.4)	21 (21.0)
BSA (m**2)				
N	117	128	108	100
Median	1.9	1.8	1.8	1.9
Min - Max	1.4 - 2.5	1.4 - 2.4	1.4 - 2.5	1.3 - 2.3

BSA = body surface area; ECOG = Eastern Cooperative Oncology Group

K-Ras Mutation Frequency in K-Ras Mutation-Positive Patients

The frequency of the 7 K-Ras mutations in the K-Ras mutation-positive population identified by the KRAS Kit is shown below. Overall, the three most common substitutions for the glycine amino acids at codons 12 and 13 were aspartic acid (34.3% and 19.2%, respectively) and valine (26.0%).

Frequency of K-Ras mutations in Mutation-Positive Patients

	Percentage of Patients	
	Cetuximab + BSC (N = 108)	BSC (N = 100)
12ALA	7.4	6.0
12ARG	0.9	1.0
12ASP	32.4	36.0
12CYS	8.3	7.0
12SER	5.6	5.0
12VAL	29.6	22.0
13ASP	15.7	23.0

Baseline Variables

Analyses of key baseline data (demographics, disease characteristics, etc.) were regenerated for each K-Ras subset. Baseline disease characteristics and characteristics that would impact testing were then compared across subsets. While there were no significant differences between subgroups, there were several unbalanced variables in the list. The imbalances are shown in the Table below.

Baseline Variables with Imbalances

Variable	K-Ras Not Evaluated	K-Ras Evaluated
Female	42.9%	33.8%
ECOG PS 2	30.3%	21.6%
Asian	17.6%	5.3%
abnormal ECG, B	38.6%	48.2%
abnormal ECG, B+C	54.8%	37.3%
Med. OS, B	4.1	4.7
Med. OS, B+C	4.8	6.5

The total list of covariates used in the multiple imputation analyses described in this report (Section D.3) are as follows:

Characteristics of tumor sample used in K-Ras testing:

- Tumor type (primary or metastatic, available for up to all patients)
- If primary, then whether right, transverse, left (including rectum) colon or other.
- If metastatic, then site (liver, lung, lymph node or other)
- Area of tumor tissue (mm^2, as continuous)
- Tumor content in sample (%, as continuous)

- Macro-dissection of the sample (yes or no)
- Necrosis score in tumor area (0, 1, 2 or 3)
- H&E staining slide evaluable (yes or no)

Handling and processing factors:

- Enrollment site (available for all patients, sites with less than 5 patients were pooled together within region)
- Region (Canada or Non-Canada, available for all patients)
- Age of sample at testing (years, as continuous)
- Sampling method (biopsy or resection)

Disease characteristics:

- Months from first histological diagnosis to randomization (as continuous)
- Primary diagnosis (rectum only vs. colon)
- Number of disease sites (>2 vs. 2)
- Presence of liver metastases (yes or no)
- EGFR maximum staining intensity (1+, 2+ or 3+)
- Number of previous chemotherapies drug class (>2 vs. 2)
- Prior radiotherapy (yes or no)
- Previous Surgeries

Patient characteristics:

- Gender (male or female)
- Race (White, Black, Asian, or Other)
- Age (years, as continuous)
- Baseline ECOG PS (0, 1 or 2)
- Baseline weight (kg, as continuous)
- K-Ras status by direct bi-directional sequencing (wild-type or mutant, only available for 394 patients)

D. Safety and Effectiveness Results

1. Safety Results

The first FDA approval action for cetuximab (Erbitux) was February 12, 2004 and was without regard to K-Ras status. Reports of serious adverse events (AEs) were collected for both study arms in the trial. The most common adverse events reported in trial CA225025 for cetuximab were skin toxicities (including rash, dry skin, pruritis, and nail changes), fatigue, infusion reactions, diarrhea, stomatitus, infections, fever, pain, dehydration, tachyarrhythmias, and insomnia.

Adverse effects that occurred in the PMA clinical study

The AE profile of cetuximab in the K-Ras wild-type subset was determined in studies that defined K-Ras status based on sequencing data, and is consistent with the known safety profile of cetuximab observed in the All Treated population based on the types, frequencies, and severity of AEs. The frequency of certain

AEs was numerically higher in the K-Ras wild-type subset compared with the K-Ras mutant subset. This difference was possibly due to the longer duration of exposure to study drug in the K-Ras wild-type subset (median of 18 weeks) compared with the K-Ras mutant subset and the All Treated population (median of 8 weeks in each population). Refer to the drug label for more information.

Cetuximab Adverse Events Based on K-Ras Status

	Number of Subjects (%)		
	C+BSC		
	K-Ras Wild Type N = 118	*K-Ras* Mutant N = 110	All Treated N = 288
Deaths within 30 days of last cetuximab treatment	23 (19.5)	21 (19.1)	59 (20.5)
On Cetuximab SAEs	54 (45.8)	49 (44.5)	134 (46.5)
AEs leading to discontinuation of cetuximab	4 (3.4)	4 (3.6)	11 (3.8)
On Cetuximab AEs of special interest (all grades)			
Hypersensitivity reaction	21 (17.8)	27 (24.5)	58 (20.1)
Rash/desquamation	112 (94.9)	92 (83.6)	253 (87.8)
Cardiovascular	38 (32.2)	20 (18.2)	86 (29.9)
On Cetuximab AEs, all grades (≥ 50%) of subjects)			
Rash/desquamation	112 (94.9)	92 (83.6)	253 (87.8)
Fatigue	104 (88.1)	95 (86.4)	248 (86.1)
Anorexia	60 (50.8)	64 (58.2)	157 (54.5)
Nausea	64 (54.2)	51 (46.4)	144 (50.0)
Dry skin	66 (55.9)	46 (41.8)	138 (47.9)
Pain other	59 (50.0)	40 (36.4)	126 (43.8)
On Cetuximab AEs, Grade 3-4 (≥ 10% of subjects)			
Fatigue	33 (28.0)	37 (33.6)	89 (30.9)
Dyspnea	16 (13.6)	10 (9.1)	36 (12.5)
Rash/desquamation	19 (16.1)	9 (8.2)	34 (11.8)
Abdominal pain	12 (10.2)	11 (10.0)	31 (10.8)
Other pain	15 (12.7)	12 (10.9)	30 (10.4)

AE = adverse event; BSC = best supportive care; C = cetuximab; SAE = serious adverse event
On-cetuximab results capture events from first dose date until 30 days after the last dose date.

2. Effectiveness Results

A. Overall Efficacy of Cetuximab, All-Randomized Population

Efficacy in the CA225025 trial was originally evaluated in patients with EGFR-expressing, metastatic colorectal cancer who had failed all prior therapies including irinotecan-containing and an oxaliplatin-containing regimen. The results of that trial in the All Randomized population are shown in the Table below.

Summary Data for Cetuximab Efficacy in the All Randomized Population

All-Randomized	**Cetuximab + BSC (n = 287)**	**BSC (n = 285)**
Median Overall Survival (months) (95z% CI)	6.14 (5.4, 6.7)	4.75 (4.2, 4.9)
Hazard Ratio (95% CI)	0.77 (0.64, 0.92)	
Log-rank test	0.0048	
Progression-fee Survival (months)	1.91	1.84
Hazard Ratio (95% CI)	0.68 (0.57, 0.81)	
Log-rank test	<0.0001	
Sensitivity Analysis for PFS (months)	1.9	1.8
Hazard ratio (95% CI)	0.58 (0.49, 0.69)	
Log-rank test	<0.0001	
Overall response rate	6.6%	0
Response duration (months)	5.5	N/A

B. <u>Efficacy of Cetuximab by K-Ras Subset, K-Ras Evaluated Population</u>

Using the K-Ras Evaluated Population, the primary and secondary analyses of cetuximab efficacy on OS and PFS were stratified to evaluate the treatment benefit within the K-Ras mutation-negative (wild type) and K-Ras mutation-positive subsets as defined by the *therascreen* KRAS Kit.

In addition, the predictive effect of the K-Ras mutation status on cetuximab efficacy (OS and PFS) was evaluated with a test for interaction between treatment group and K-Ras mutation status, using a stratified Cox proportional hazards model with treatment group, K-Ras mutation status, and the interaction between the two as factors.

Overall Survival by K-Ras Population

In the K-Ras Evaluated Population, OS efficacy results by K-Ras mutation-negative (wild-type) and mutation-positive subsets are summarized in Table below.

Overall Survival	K-Ras Mutation-negative (Wild-type)		K-Ras Mutation-positive	
	Cetuximab + BSC	BSC	Cetuximab + BSC	BSC
	N = 117	N= 128	N= 108	N= 100
Median (months)	8.6	5.0	4.8	4.6
(95% CI)	(7.0, 10.3)	(4.3, 5.7)	(3.9, 5.6)	(3.6, 4.9)
Hazard ratio (95% CI)	0.63 (0.47, 0.84)		0.91 (0.67, 1.24)	
Log-rank p-value	<0.0017		0.5507	
Interaction p-value	<0.0699			

For the K-Ras Mutation-negative (Wild-type) Population, a statistically significant reduction in the risk of death was observed with use of cetuximab + BSC. Median survival time (95% % confidence interval [CI]) was 8.6 (7.0, 10.3) months in the cetuximab + BSC group and 5.0 (4.3, 5.7) months in the BSC group. The OS hazard ratio of cetuximab + BSC over BSC was 0.629, indicating reduced risk of death for subjects randomized to cetuximab + BSC. The 95% CI was (0.47, 0.84). The hazard ratio was less than 1 with high statistical significance (p value 0.0017).

For the K-Ras Mutation-positive Population, a small, insignificant difference in OS was observed between the two treatment groups. Median survival time (95% CI) was 4.8 (3.9, 5.6) months in the cetuximab + BSC group and 4.6 (3.6, 4.9) months in the BSC group. The hazard ratio was 0.91 with 95% CI (0.67, 1.24), and was not significantly less than 1 (p value 0.5517).

Fn the K-Ras mutation-negative (wild-type) subset, overall survival rates based on Kaplan-Meier estimates at months 6 and 12 were higher for the cetuximab + BSC group than the BSC group. In the K-Ras mutation-positive subset, this advantage was not observed (see Figures 1 and 2 below).

The difference between OS hazard ratios 0.629 and 0.911 for the K-Ras mutation-negative (wild-type) and mutation-positive subsets, although large, was not quite statistically significant. The two-sided p value for interaction between K-Ras mutation status and treatment group was greater than 0.05 (0.0699 using maximum likelihood, 0.0703 using Cox model). Thus, KRAS Kit mutation status was not quite statistically significant as a predictor of OS efficacy.

Figure 1: Kaplan Meier Plot of Overall Survival- K-Ras Mutation-negative (Wild-type)

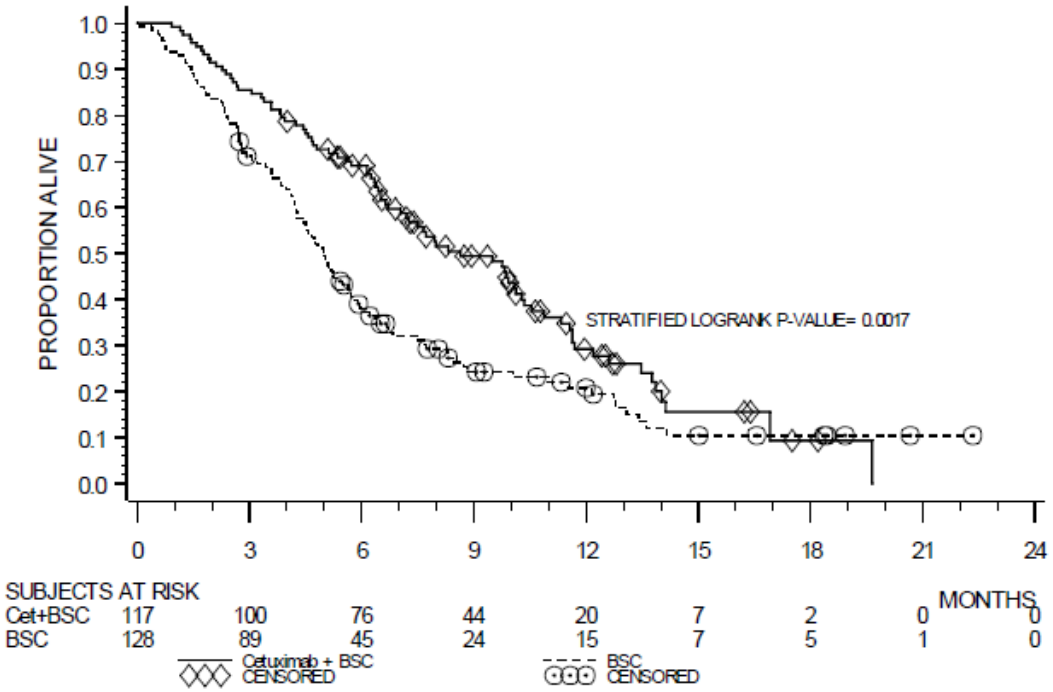
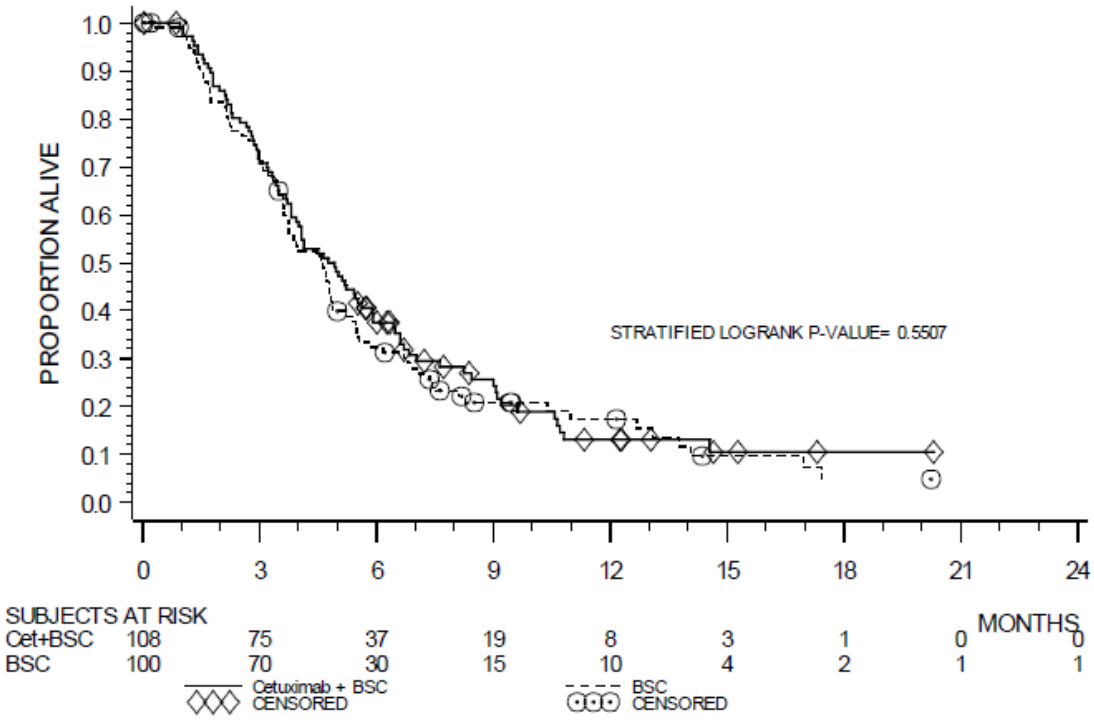


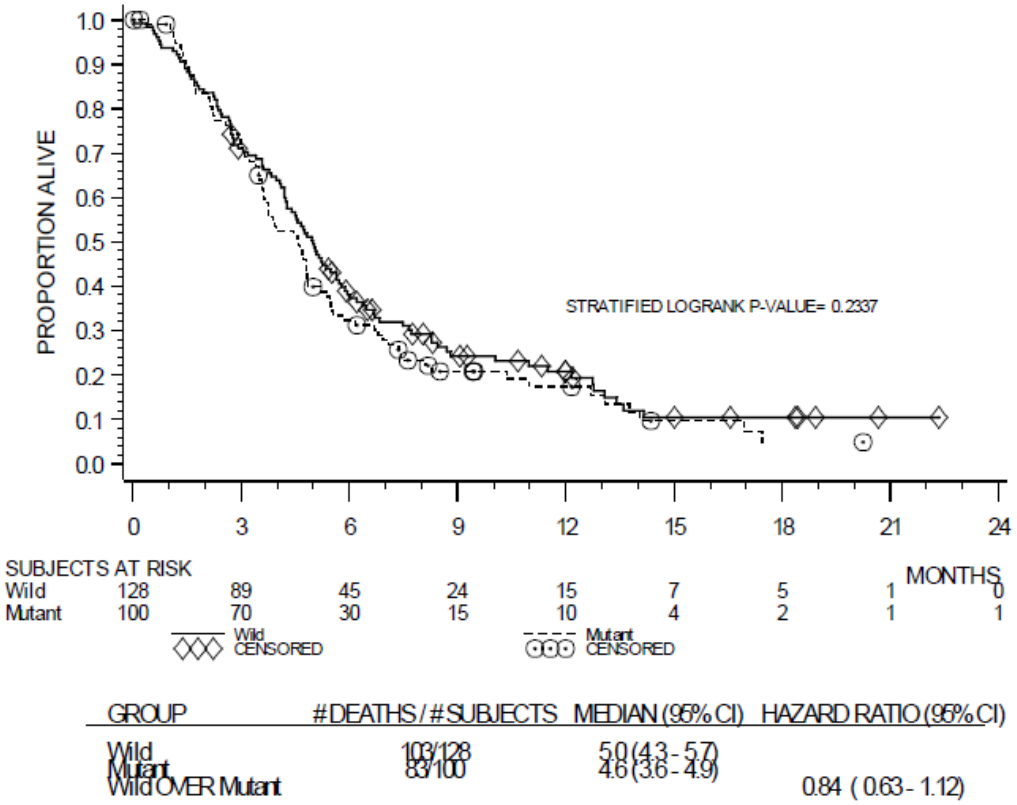
Figure 2: Kaplan Meier Plot of Overall Survival- K-Ras Mutation-positive



K-Ras Prognostic Effect, OS

K-Ras mutation status was not observed to be prognostic for overall survival in the BSC group (see Figure 3 below).

Figure 3: Kaplan-Meier plot of OS by K-Ras Mutation Status (K-Ras Evaluated, BSC group.)



Progression Free Survival by K-Ras Population

In the K-Ras Evaluated Population, PFS efficacy results by K-Ras mutation-negative (wild-type) and K-Ras mutation-positive subsets are summarized in the Table below.

Progression Free Survival	K-Ras Mutation-negative (Wild-type)		K-Ras Mutation-positive	
	Cetuximab + BSC	BSC	Cetuximab + BSC	BSC
	N = 117	N= 128	N= 108	N= 100
Median (months)	3.8	1.9	1.8	1.8
(95% CI)	(3.6, 5.4)	(1.8, 2.0)	(1.7, 1.8)	(1.7, 1.9)
Hazard ratio (95% CI)	0.42 (0.32, 0.56)		1.12 (0.84, 1.49)	
Log-rank p-value	<0.0001		0.4276	
Interaction p-value	<0.0001			

For the K-Ras Mutation-negative (Wild-type) Population, a statistically significant improvement in PFS was observed with use of cetuximab + BSC. Median (95% CI) PFS time was 3.8 (3.6, 5.4) months in the cetuximab + BSC group compared with 1.9 (1.8, 2.0) months in the BSC group. The PFS hazard ratio of cetuximab + BSC over BSC was 0.42, indicating improved PFS for subjects randomized to cetuximab + BSC. The 95% CI was (0.32, 0.56). The PFS hazard ratio was less than 1 with high statistical significance (p value < 0.0001).

For the K-Ras Mutation-positive Population, an insignificant difference in PFS was observed with the use of cetuximab. Median (95% CI) survival time was 1.8 (1.7, 1.8) months in the cetuximab + BSC group and 1.8 (1.7, 1.9) months in the BSC group. The hazard ratio was 1.12 with 95% CI (0.84, 1.49). It was not significantly greater than 1 (p value 0.5517).

In the K-Ras mutation-negative (wild-type) subset, PFS rates based on Kaplan-Meier estimates were greater at all time-points for the cetuximab + BSC group compared with the BSC group. In the K-Ras mutation-positive subset, the rates were similar for each treatment group (see Figures 4 and 5 below.)

The difference between PFS hazard ratios 0.42 and 1.12 for the K-Ras wild-type and mutant subsets was statistically significant. The 2-sided p value for interaction was less than 0.05 (< 0.0001 using either maximum likelihood or the Cox model). Thus, statistically, KRAS Kit mutation status was a significant predictor of PFS efficacy.

Figure 4: Kaplan-Meier Plot of PFS - K-Ras Mutation-Negative (Wild-type)

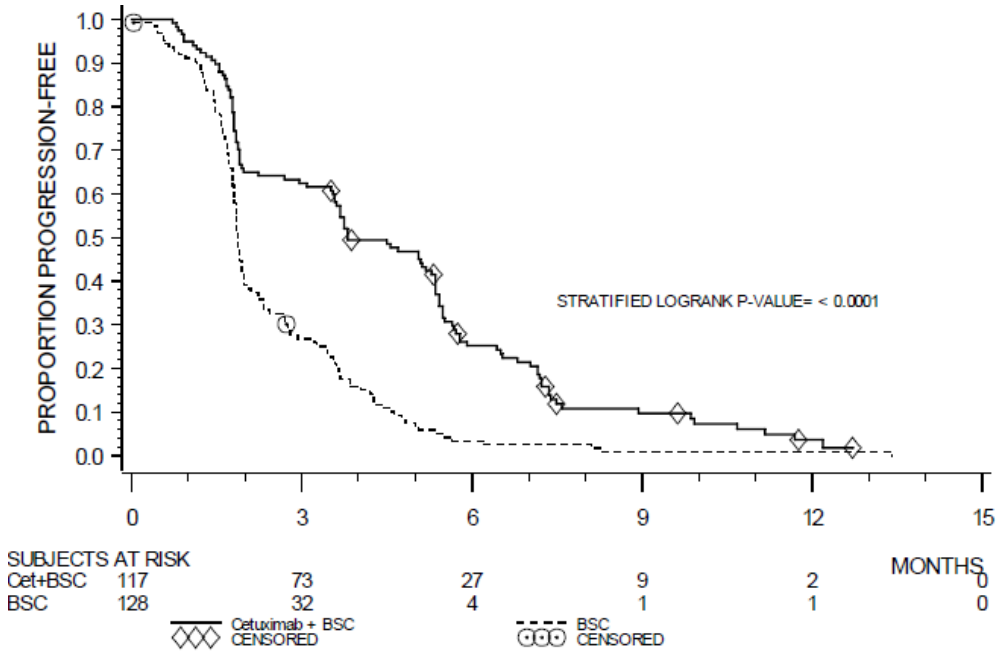
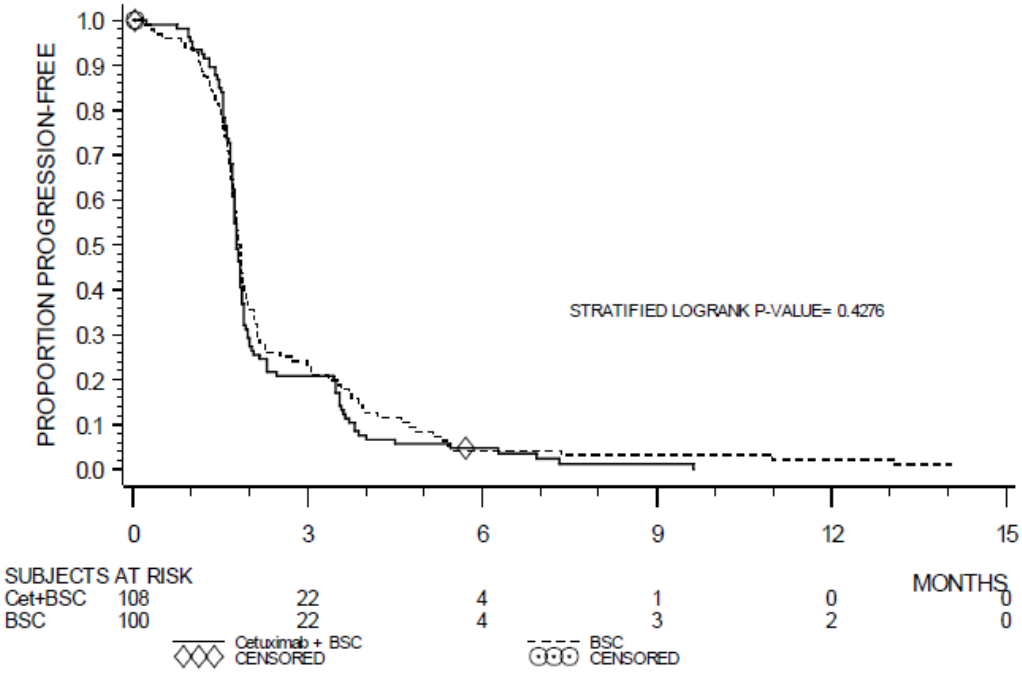


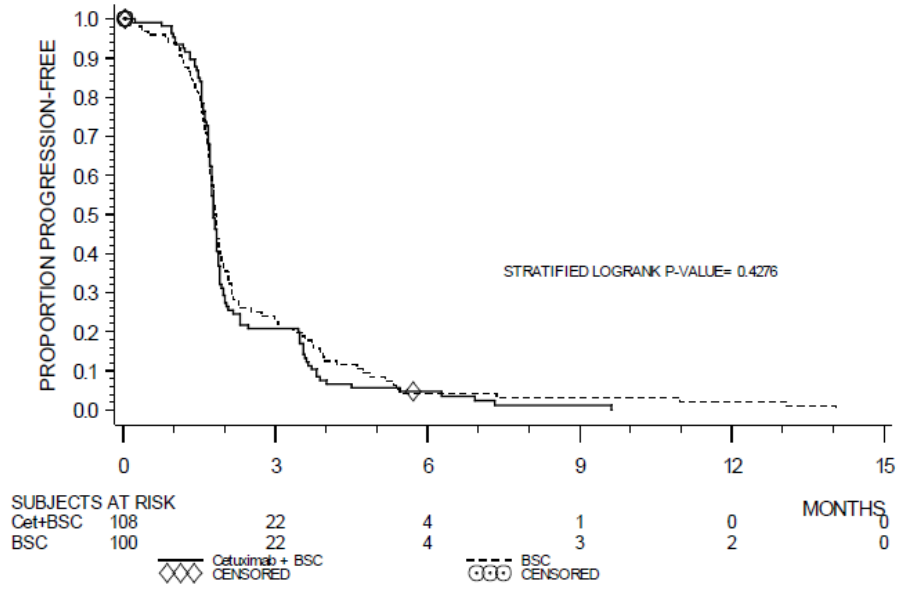
Figure 5: Kaplan-Meier Plot of Progression-free Survival - K-Ras Mutation-Positive



K-Ras Prognostic Effect, PFS

K-Ras mutation status was not observed to be prognostic for longer progression-free survival in the BSC group (see Figure 6 below).

Figure 6: Kaplan-Meier Plot of PFS by K-Ras Mutation Status (K-Ras Evaluated, BSC group.)



GROUP	#EVENTS / #SUBJECTS	MEDIAN (95% CI)	HAZARD RATIO (95% CI)
Cetuximab + BSC	105/108	1.8 (1.7 - 1.8)	
BSC	96/100	1.8 (1.7 - 1.9)	
Cetuximab + BSC OVER BSC			1.12 (0.84 - 1.49)

3. Sensitivity Analyses

Efficacy of Cetuximab by K-Ras Subset, All-Randomized Population: Sensitivity of OS Efficacy to Missing K-Ras Results

K-Ras test results were obtained for 453 of the 572 (79.2%) patients in the All-Randomized Population. Because the percentage is less than 90%, the minimum recommended by the FDA Oncology Drugs Advisory Committee (16-Dec-2008 meeting) (2), a broad range of sensitivity analyses were conducted to address the potential impact of missing K-Ras results on the primary efficacy endpoint of OS. Missing K-Ras results were imputed as wild-type or mutant to obtain a set of completed data for the All-Randomized Population, on which OS efficacy by K-Ras status and the predictive effect of the K-Ras status on OS were analyzed. The sensitivity analyses are measures of robustness to the impact of missing K-Ras results on conclusions made regarding OS efficacy in the K-Ras Evaluated Population.

Imputation of missing K-Ras results were based on three pre-specified types of methods:

- Imputation Based on a Pre-specified Random Mechanism – Simulation
- Imputation Based on a Pre-specified Random Mechanism – Multiple Imputation (MI) based on observed covariates (two models).
- Deterministic Imputation Based on Extreme Scenarios

For the first two methods, multiple completed datasets were obtained and analyzed, with results combined (Rubin's method), to account for imputation uncertainty. The second, MI method assumes that K-Ras status is *missing at random* (i.e., probability that a test result is missing is a function of observed data, not missing data.)

The imputation models all conferred a significant treatment effect in the K-Ras wild-type population except for the worst case extreme scenario D1 (See table below). For that worst case scenario, missing K-Ras status was imputed such that patients imputed to be wild-type had a huge deleterious hazard ratio of 8.15. None of the imputation models except for favorable scenarios B2, C2, and D2 conferred a significant predictive effect of K-Ras status on cetuximab efficacy (interaction p value > 0.05). The results of the imputation studies are summarized below.

OS Efficacy, by Imputation Model for Missing K-Ras status, All Randomized Population

	K-Ras Mutation-negative (wild-type)		K-Ras Mutation-positive		
Scenario for Imputing K-Ras Status	**Hazard Ratio**	**(95% CI)**	**Hazard Ratio**	**(95% CI)**	**Interaction p-value**
Simulation Method(0)†	0.666	(0.511, 0.869)	0.888	(0.671, 1.176)	0.1525
MI Model 1: Uses a pre-specified set of variables(1)†*	0.667	(0.511, 0.871)	0.875	(0.660, 1.161)	0.1822
MI Model 2: Uses model 1 variables plus bi-directional sequencing K-Ras result(2)†*	0.668	(0.512, 0.872)	0.878	(0.665, 1.158)	0.1658
Extreme Scenarios for Imputing K-Ras status, OS, All Randomized Patients(3)					
A1	0.681	(0.539, 0.861)	0.911	(0.673, 1.235)	0.1091
A2	0.629	(0.470, 0.843)	0.868	(0.683, 1.104)	0.1037
B1	0.699	(0.553, 0.883)	0.877	(0.647, 1.189)	0.2420
B2	0.607	(0.453, 0.814)	0.895	(0.704, 1.138)	0.0454
C1	0.717	(0.557, 0.924)	0.826	(0.629, 1.084)	0.3572
C2	0.601	(0.464, 0.778)	0.960	(0.735, 1.253)	0.0162
D1	0.815	(0.633, 1.050)	0.663	(0.505, 0.871)	0.2924
D2	0.529	(0.408, 0.686)	1.220	(0.931, 1.597)	<.0001

†To account for the between imputation variability, multiple completed data sets were obtained from an imputation model and combined using Rubin's multiple imputation method (PROC MIANALYZE in SAS.). The number of completed datasets was determined such that relative efficiency of parameter estimates exceeded 99%. Analyses were based on a Cox model of OS stratified by randomization factor ECOG status (PS 0-1 vs. 2) that included treatment group, K-Ras mutation status, and their interaction as factors.

(0) Subjects with missing *K-Ras* status were randomly imputed as K-*Ras* wild-type or mutant according to the proportion of subjects with *K-Ras* wild-type tumors in the *K-Ras* Evaluated population (54%). Fifteen completed datasets were generated and combined by Rubin's MI method.

(1) This model excluded K-Ras status by bi-directional sequencing as an imputation variable. Sixteen completed datasets were generated with results combined by Rubin's MI method.

(2) This model included K-Ras status by bi-directional sequencing as an imputation variable. Thirteen completed datasets were generated with results combined by Rubin's MI method.

(3)
- Scenario A1: Impute all missing status as wild-type
- Scenario A1: Impute all missing status as wild-type
- Scenario A2: Impute all missing status as mutant
- Scenario B1: Impute missing status as wild-type if patient died, else mutant
- Scenario B2: Impute missing status as mutant if patient died, else wild-type
- Scenario C1: Impute missing status as wild-type if patient's OS time was "short" among the shortest 54% OS time), else mutant
- Scenario C2: Impute missing status as wild-type if patient's OS time was "long" (among the longest 54% OS time), else mutant
- Scenario D1: For cetuximab + BSC (BSC alone) group, impute wild-type if patient's OS time was among the 54% with the "shortest" ("longest") times, else mutant
- Scenario D2: For cetuximab + BSC (BSC alone) group, impute wild-type if patient's OS time was among the 54% with the "longest" ("shortest") times, else mutant

* For the MI models, a list of covariates that included characteristics of tumor sample used in *K-Ras* testing; handling and processing; disease characteristics; patient characteristics. The complete list of variables is given above in Section C of this report.

To address variables that were not included in the primary multiple imputation analysis of prespecified variables, in particular "baseline abnormalities on ECG," analysis was conducted on all the variables along with the pre-specified variables included in the initial multiple imputation model, including "concomitant anti-cancer treatment before progression." The results were consistent with the earlier analyses and are shown below.

Overall Survival after Imputation of Missing K-Ras Data through Multiple Imputations - All Randomized Subjects (by K-Ras Mutation Status)

	K-Ras Mutation-negative (wild-type)		**K-Ras Mutation-positive**		
Scenario for Imputing K-Ras Status	**Hazard Ratio**	**(95% CI)**	**HR**	**(95% CI)**	**Interaction p-value**
Primary model using prespecified variables	0.667	(0.511, 0.871)	0.875	(0.660, 1.161)	0.1822
Primary model with abnormal ECG as baseline and pre-specified variables	0.674	(0.511, 0.971)	0.875	(0.660, 1.120)	0.2283
Primary model with abnormal ECG at baseline, concomitant anti-cancer treatment before progression flag, OS time, censoring indicator and pre-specified variables	0.664	(0.510, 0.865)	0.896	(0.675, 1.190)	0.1513
Primary model with abnormal ECG at baseline, concomitant anti-cancer treatment before progression flag, OS time, censoring indicator, treatment indicator, interaction of OS time*treatment indicator, interaction of censoring indicator*treatment indicator and pre-specified variables	0.647	(0.500, 0.837)	0.928	(0.701, 1.230)	0.0641

XI. SUMMARY OF SUPPLEMENTAL CLINICAL INFORMATION

Tumor samples from patients in the CA225025 trial were originally assessed by bi-directional sequencing to provide evidence of the impact of K-Ras as a factor in response to cetuximab. These results were first published in 2008 and were used as the basis for the planned studies of cetuximab efficacy in K-Ras subsets with the KRAS Kit. In the all Randomized population, 375 (65.5%) of patients had K-Ras results for both the KRAS Kit and bi-directional sequencing. A comparison of the original sequencing results and the KRAS Kit results are shown below.

	Bi-Directional Sequencing Results									
KRAS Kit Result(a)	12ALA	12ASP	12ARG	12CYS	12SER	12VAL	13ASP	Wild-type	Other (b)	Total
12ALA	9	-	-	-	-	-	-	4	1	14
12ASP	-	49	-	-	2	2	-	6	12	71
12ARG	-	-	1	-	-	-	-	6	-	2
12CYS	1	2	_	7	-	-	-	5	1	16
12SER	-	1	-	-	5	-	-	-	5	11
12VAL	-	-	-	-	2	35	-	12	5	54
13ASP	1	-	-	-	-	-	20	10	9	40
Negative	-	5	-	-	4	6	1	184	45	245
Invalid	-	-	-	-	2	2	-	1	2	7
Not available	-	2 (c)	1	1	1	2 (c)	-	7	99	113
Total	11	59	2	8	16	47	21	230	179	573

(a) If mutation type by bi-directional sequencing had more than one mutation, a match was considered if the KRAS Kit detected either. There were seven cases where more than one mutation was detected by sequencing.

(b) Other refers to any other mutation not detected by the KRAS Kit (there was one 13Cys/13VAL) and not available for testing.

(c) One sample was counted twice (because) it had two mutations per sequencing.

Agreement between the two methods in terms of mutation-positive vs. no mutation detected is shown below. No-mutation detected by bi-directional sequencing indicates the samples did not have any of the 7 mutations detected by the KRAS Kit (i.e., a sample having a mutation not detected by the KRAS Kit is included in this subgroup).

	Bi-directional Sequencing			
KRAS Kit	No Sample	No mutation detected	mutation-positive	Total
Not evaluated	97*	7	7	113
Invalid	2	1	4	7
no-mutation detected	45	185	16	245
mutation-positive	33	38	137	208
Total	177	231	164	572

*One sample designated 'Other' in the concordance table was 13CYS/13VAL by sequencing and mutation-not-detected by the KRAS Kit; One sample that was 12ASP/12VAL by sequencing and not evaluated by KRAS Kit was counted twice in the table above by sponsor and was counted once in this table.

- Positive percent agreement 137/153 = 89.5%
- Negative percent agreement 185/223 = 82.9%
- Overall percent agreement 322/376 = 85.6%

The majority of disagreement between the 38 samples determined to be no mutation detected by Sanger and mutation-positive by KRAS Kit was attributed to the enhanced

sensitivity of the KRAS Kit assay when compared to Sanger. This is supported by two analytical performance studies designed to measure concordance where the samples are first-macrodissected for bi-directional sequencing to improve tumor content. The samples were from the intended use population. In the study "Comparison to Analytical Reference Method," the overall percent agreement was significantly higher (96.4% and See agreement table below)

Accuracy Studies 1 and 2 (from Non-clinical performance section)

	Percentage	
Measurement of Agreement	**Study 1**	**Study 2**
Overall percent agreement	96.3%	96.4%
Percent positive agreement	96.3%	99.1%
Percent negative agreement	96.4%	94.3%

While the agreement is less than the agreement observed with procured samples, the sponsor indicated that the original bi-directional studies were not conducted with the intent to support a PMA (e.g., performed with acceptance criteria and macrodissected to improve the sensitivity of the Sanger sequencing) and, new sections were extracted for this evaluation. Therefore, due to the heterogeneous nature of tumor specimens, it isn't clear that the content of the sample evaluated by either method was the same in tumor content and mutant content. A breakdown of putative reasons for discordance included non-consecutive sampling, lack of macro-dissection (for bi-directional sequencing), low mutant content, and variable sequence results with bi-directional.

Overall, the conclusion was that the two procured samples sets were supportive of the QIAGEN kit accuracy, and the accuracy of this data was sufficient to support selecting patients for treatment when patient CRC tumor specimens are evaluated by the KRAS Kit, based on the efficacy analyses.

XII. PANEL MEETING RECOMMENDATION AND FDA'S POST-PANEL ACTION

In accordance with the provisions of section 515(c)(2) of the act as amended by the Safe Medical Devices Act of 1990, this PMA was not referred to the Immunology Panel, an FDA advisory committee, for review and recommendation.

XIII. CONCLUSIONS DRAWN FROM PRECLINICAL AND CLINICAL STUDIES

A. Effectiveness Conclusions.

The clinical benefit of the *therascreen* KRAS RGQ PCR Kit was demonstrated in a retrospective analysis of efficacy and safety in patients without K-Ras mutations detected by the KRAS Kit. Overall, a statistically significant efficacy benefit for cetuximab + BSC vs. BSC was observed in the subset of patients with K-Ras mutation-negative (wild-type) tumors, whereas no such benefit was observed in the subset of patients with K-Ras mutation-positive tumors. Results in the K-Ras mutation-negative (wild-type) subset were consistent across both efficacy endpoints

of OS and PFS. Results from the sensitivity analyses consistently demonstrate an improvement in overall survival in the K-Ras mutation-negative (wild-type) group, and no meaningful improvement in the K-Ras mutation-positive group.

B. Safety Conclusions

The risks of the device are based on nonclinical laboratory studies to support PMA approval as described above, as well as data collected in a clinical studies conducted to support cetuximab approval. The safety profile of cetuximab in the subset of subjects without K-Ras mutations in codons 12 and 13 is consistent overall with that reported for the All Treated population and with the known safety profile of cetuximab. Failure of the device to perform as expected or failure to correctly interpret test results may lead to incorrect K-Ras test results, and consequently improper patient management decisions in colorectal cancer treatment. A false positive test result may lead to cetuximab treatment being withheld from a patient who might have benefitted. A false negative test result may lead to cetuximab treatment being administered to a patient who is not expected to benefit, and potentially any adverse side effects associated with treatment.

C. Benefit-Risk Conclusions

Colorectal cancer is the third most commonly diagnosed cancer and the third-leading cause of cancer-related death in both men and women in the United States (American Cancer Society [ACS] 2011). For 2011, the American Cancer Society predicts approximately 141,210 new cases and 49,380 deaths in the United States. Deaths from CRC account for about 9% of all cancer deaths (ACS 2010). The incidence and death rates for CRC increase with age, with over 90% of new cases and deaths occurring in patients (subjects) 50 years and older (ACS 2011). Overall, 1- and 5-year relative survival rates for patients with CRC are 83% and 67%, respectively (ACS 2010, 2011). When CRC is detected at an early, localized stage, the 5-year survival rate is 90%; however, only 39% of patients with CRC are diagnosed at this stage. After the cancer has spread regionally to involve adjacent organs or lymph nodes, the 5-year survival rate drops to 70%. When the disease has spread to distant organs (metastatic disease), the 5-year survival rate is 12%.

The probable benefits of the device are based on data collected in the clinical study conducted to support PMA approval as described above. The clinical benefit of the *therascreen* KRAS RGQ PCR Kit was demonstrated in a retrospective analysis of efficacy and safety data obtained from patients with metastatic colorectal cancer from Study CA225025 determined to be K-Ras mutation-negative (wild-type) by the KRAS Kit (reported as no mutation detected). The duration of the effect for overall survival was 8.6 months in the K-Ras mutation-negative (wild-type) subset treated with cetuximab plus best supportive care compared to 5.0 months for K-Ras mutation-negative (wild-type) patients who received best supportive care alone. In contract, the K-Ras mutation-positive subset was 4.8 months compared to 4.6 months respectively. A benefit in progression free survival was also observed in the K-Ras mutation-negative (wild-type) subset.

The risks of the KRAS Kit are associated the potential mismanagement of patients resulting from false results of the test. Failure of the device to perform as expected or failure to correctly interpret test results may lead to incorrect K-Ras test results, and consequently improper patient management decisions in colorectal cancer treatment. A false positive test result may lead to Erbitux® (cetuximab) treatment being withheld from a patient who might have benefitted. A false negative test result may lead to Erbitux (cetuximab) treatment being administered to a patient who is not expected to benefit, and potentially any adverse side effects associated with treatment. The device is a key part of diagnostic evaluation for colorectal cancer in decisions regarding treatment with cetuximab. There is currently no FDA approved test for the selection of candidate metastatic CRC patients for treatment with cetuximab.

In conclusion, given the available information above, the data support the use of the *therascreen* KRAS RGQ PCR Kit as an aid in the identification of CRC patients for Erbitux (cetuximab) treatment based on a KRAS Kit no mutation detected test result, and the probable benefits outweigh the probable risks.

D. Overall Conclusions

The data in this application support the reasonable assurance of safety and effectiveness of this device when used in accordance with the indications for use. Data from CA225025 support the utility of the *therascreen* KRAS RGQ PCR Kit as an aid in the identification of patients with metastatic CRC s for treatment with Eribtux (cetuximab). Cetuximab + BSC demonstrated significant improvement in OS compared with BSC in subjects with K-Ras mutation-negative (wild-type) tumors identified with the *therascreen* KRAS RGQ PCR Kit test. No meaningful difference in OS between treatment groups was observed in the K-Ras mutation-positive subgroup. Sensitivity analyses assessing the impact of missing K-Ras evaluation on OS showed results that were consistent with the primary analysis. Results for OS were supported by significant improvements in PFS in the cetuximab + BSC group vs. the BSC group in subjects with K-Ras mutation-negative (wild-type) tumors.

XIV. CDRH DECISION

CDRH issued an approval order on July 6, 2012. The final conditions of approval can be found in the approval order.

The applicant's manufacturing facilities were inspected and found to be in compliance with the device Quality System (QS) regulation (21 CFR 820) on May 22, 2012.

XV. APPROVAL SPECIFICATIONS

Directions for use: See device labeling

Hazards to Health from Use of the Device: See Indications, Contraindications, Warnings, Precautions, and Adverse Events in the device labeling. Refer to the drug label for cetuximab (Erbitux®) for additional information related to the use of the drug.

Post-approval Requirements and Restrictions: See approval order.

XVI. REFERENCES.

1. Karapetis CS, Khambata-Ford S, Jonker DJ, et al. *K-Ras* mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med*. 2008; 359:1757-1765.
2. Mack, GS. FDA holds court on *post hoc* data linking KRAS status to drug response *Nature Biotech*, 2009, 27(2), 110-112.